THE INTEGRATED FUNCTION OF CARDIAC, SKELETAL, AND VASCULAR SMOOTH MUSCLE: LIMITATIONS TO OXYGEN TRANSPORT AND UTILIZATION IN HEALTHY AGING AND DISEASE

by

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ABSTRACT

The purpose of this dissertation was to investigate the integration of cardiac, skeletal, and vascular smooth muscle in the process of oxygen (O_2) transport and utilization. The goal of the first study was to examine the vasomotor function of human skeletal muscle feed arteries (SMFAs), utilizing pressure myography, with a focus on the impact of age. In this study, we demonstrated that in response to an increased shear stress, the SMFAs likely contribute to the regulation of vascular resistance/conductance *in vivo*. Furthermore, aging likely blunts the capacity to delivery O_2 due to reduced endothelial function in these SMFAs identified by attenuated kinetics of vasodilation and maximum vasodilatory capacity. Additionally, this attenuated vascular function was associated with a reduction in the shear-induced activation of eNOS, and elevated free radical production in SMFAs with age. The second study using normal healthy donor hearts (HdH) as a reference, sought to examine the impact of heart failure (HF) etiology on mitochondrial function in the pathology. Specifically, this study examined cardiac muscle mitochondrial function and free radical production in patients with ischemic HF (iHF) in comparison to that of patients with non-ischemic HF (niHF). Both mitochondrial quality and quantity were compromised in HF compared to HdH. Interestingly, a lower tissue mass specific oxidative phosphorylation (OXPHOS) capacity was documented in iHF compared to niHF, which was predominantly due to reduced mitochondrial content. However, increased non-phosphorylating respiration, and elevated mitochondrial derived free

radical production, as well as an attenuated efficiency of OXPHOS in iHF compared to niHF suggests an etiology-specific reduction in intrinsic mitochondrial function in iHF. However, increased non-phosphorylating respiration, and elevated mitochondrial-derived free radical production, as well as an attenuated efficiency of OXPHOS in iHF compared to niHF, suggests an etiology specific reduction in intrinsic mitochondrial function in iHF. Therefore, this study identified HF etiology as an important contributor to the functional abnormalities associated with HF. The third study examined the characteristics and respiratory function of mitochondria in cardiac, skeletal, and vascular smooth muscle. Tissue-specific mitochondrial OXPHOS normalized by citrate synthase activity (CSA) was similar in cardiac, skeletal, and smooth muscle; however, there were significant muscle-specific differences in both non-phosphorylating respiration and efficiency of OXPHOS. These findings suggest that different muscle tissues have distinct intrinsic mitochondrial function which may influence the efficiency of OXPHOS and potentially free radical production. In summary, this set of studies has identified novel mechanisms underlying blunted O_2 supply and utilization often associated with aging and disease in humans.

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CHAPTER 1

INTRODUCTION

The oxygen (O_2) cascade, a stepwise movement of O_2 from atmospheric air to the mitochondria of muscle and other organs, starts in the lung where O_2 is transferred from air to blood. The O_2 rich blood is then propelled by the rhythmic pumping of the heart through blood vessels, steered by changes in vascular resistance to deliver O_2 to metabolically active tissue [1, 2]. Thus, especially during physical activity, the cardiovascular system, including the heart and blood vessels, work in concert to transfer O_2 rich blood to skeletal muscle, where the mitochondria consume O_2 to produce energy in the form of adenosine triphosphate (ATP) by oxidative phosphorylation (OXPHOS). Therefore, cardiac muscle, the vascular smooth muscle, skeletal muscle, and the integration of these three muscle systems, are major components of the O_2 transport and utilization systems [1].

Although the O_2 cascade has been studied in earnest for many decades, numerous mechanisms and the implications of alterations in function due to aging and disease, such as attenuated convective O_2 transport capacity and apparent mitochondrial dysfunction, remain poorly understood. Therefore, the purpose of this dissertation was to investigate three topics related to O_2 transport and utilization linked by the integrative function of the heart, skeletal, and smooth muscle in health and disease. Specifically, the first study focused upon how aging impacts the vasodilatory capacity of human skeletal muscle feed arteries (SMFA). The second study examined the impact of heart failure (HF) and more specifically the effect of HF etiology on mitochondrial function in cardiac muscle. Finally, the third study comprehensively assessed and compared mitochondrial respiratory function in cardiac, skeletal, and, for the first time, smooth muscle. Therefore, by examining components of these three muscle systems, the overarching goal of this

dissertation was to contribute significantly to our understanding of the limitations to O_2 transport and utilization in health and disease**.**

Blood flow regulation in the peripheral vasculature and the impact of age

In addition to the significant role that the heart plays in the convective delivery of O_2, predominantly the maintenance of blood pressure, the vasomotor function of blood vessels, which regulates resistance, is the major determinant of blood flow distribution. Indeed, blood vessels act as an important "middle man" in the transfer of oxygenated blood from the heart to areas of high O_2 demand, such as skeletal muscle [2]. Therefore, especially during exercise, optimal vasodilatory function of blood vessels is essential to meet skeletal muscle O_2 demand [2]. These convective components of cardiovascular O_2 delivery system, the heart and blood vessels, can be regulated by many factors, including neural, hormonal, and local mechanisms [1]. At the onset of exercise, neural control is often considered the primary blood flow regulator, then hormonal regulation and local control predominate. However, these mechanisms work both independently and interactively in response to significantly increased O_2 demand in the transition from resting to working skeletal muscle [3]. Interestingly, previous studies suggested that the impairment of local vasodilatory mechanisms at the onset of exercise may play a critical role in limiting O_2 transport and therefore exercise capacity in both healthy aging and disease [4-6].

The impaired local vasodilatory mechanisms, as exemplified by diminished vascular function with age, cause a temporal O_2 delivery to O_2 demand mismatch across the rest to exercise transition, which has been documented in both animals and humans [7,

8]. There are several mechanisms responsible for this age-related reduction in O_2 delivery to skeletal muscle, including fewer SMFAs, impaired endothelium-dependent vasodilation mediated by reduced nitric oxide (NO) bioavailability, and alternations in blood vessel structure [8-11]. Although the role of arterioles in the regulation of blood flow distribution within skeletal muscle with age has been studied intensively [8, 9, 12], the regulatory function of SMFAs, directly upstream from the arterioles, and the effects of age on these vessels have yet to be comprehensively assessed. Therefore, the purpose of the first study of this dissertation was to investigate the impact of age on the vasodilatory capacity of SMFAs. We tested four hypotheses: First, both young and old SMFAs would exhibit significant vasodilatory capacity stimulated by flow-induced shear stress, acetylcholine (ACh), and sodium nitroprusside (SNP). Second, the endothelium-mediated vasodilatory response (flow-induced shear stress and ACh) would be attenuated in the old compared to the young, whereas the endothelium-independent vasodilatory response (SNP) would be similar between groups. Third, the flow-induced vasodilation kinetics will be slowed with less activation of endothelial nitric oxide synthase (eNOS) in the old compared to the young. Finally, free radical levels will be greater in the old compared to the young.

The impact of HF on mitochondrial respiration in cardiomyocytes

Mitochondria are ubiquitous organelles constituting up to 40% of cytoplastic volume in mammalian tissues such as kidney, liver, and heart. Consequently, it has been estimated that there are upwards of 10 million billion mitochondria in a human adult [13]. In addition to an essential role in ATP generation, mitochondria are important

components of bioenergetic pathways, cellular redox homeostasis, regulation of calcium signaling, and apoptosis. Interestingly, as a consequence of OXPHOS, mitochondria are also recognized as a major source of free radicals which may result in cellular damage [14-18], and, thus, mitochondria have been implicated in the pathophysiology of aging and disease in several organs, including the brain, heart, skeletal, and smooth muscle [14, 19, 20] .

OXPHOS involves the oxidation of fuels (glucose, free fatty acids, lactate, etc.) to make ATP which is then hydrolyzed to generate the energy which drives endergonic reactions essential for life. Therefore, the rate of OXPHOS and ATP hydrolysis is tightly matched to ATP demand and supply [21, 22]. Briefly, mitochondrial respiration involves the reduction of NADH and $FADH_2$ in complex I and complex II, respectively, then these complexes transfer electrons to complex III through coenzyme Q. Complex IV receives electrons from complex III, and then reduces (consumes) O_2 by adding electrons to produce H_2O. In this electron transport process, hydrogen ions are pumped out from mitochondrial matrix to the intermembrane space and then, eventually, hydrogen ions are funneled back to the matrix though ATP synthase (complex V), providing the energy to produce ATP from the reaction between ADP and inorganic phosphate [22].

Characteristics of mitochondrial function can be assessed *in vitro* by measuring tissue O_2 consumption rate in a series of conditions that challenge the different complexes and evoke various levels of respiration. Specifically, 1) Complex I state 2 respiration, the non-phosphorylating resting state that provides an index of proton leak, assessed in the presence of malate + glutamate, 2) Complex I, state 3 respiration, the ADP-activated state of OXPHOS, assessed in the presence of glutamate + malate + ADP,

3) Complex I+II, state 3 respiration, assessed in the presence of glutamate + malate + ADP + succinate, 4) Complex II, state 3 respiration, assessed in the presence of glutamate + malate + ADP + succinate + rotenone 5) Complex IV respiration, assessed by the blocking of Complex 3 (antimycin A) and Complex V (oligomycin) followed by the addition of TMPD + ascorbate [23].

Utilizing these approaches, impaired skeletal muscle mitochondrial function and increased mitochondrial free radical production have been reported in numerous studies as a function of aging and disease [24-26]. However, mitochondrial function in human cardiac muscle is less well studied due to the limited opportunity to obtain cardiac muscle from healthy and diseased individuals. Furthermore, a limited number of investigations utilizing human heart tissue obtained from diseased hearts during routine surgeries have yet to clearly elucidate the impact of heart failure on mitochondrial function due to confounding factors such as varied etiology, severity of disease, and comorbidities.

Mitochondria are dynamic and plastic organelles that demonstrate both structural and functional adaptations in response to specific stimuli, such as the increased energy demands associated with exercise training [27, 28]. However, alterations in mitochondrial function arising from changes in substrate availability and disease impair mitochondrial respiratory function [29-32]. Common characteristics of negatively impacted mitochondrial function are an impaired OXPHOS (decreased Complex I, Complex II, and I+II, state 3 respiration), increased O_2 consumption coupled with decreased ATP production (increased Complex IV activity, but with lower ATP production), and increased free radical production (associated with Complex I, state 2 respiration).

The heart is an organ with a high metabolic demand and is, therefore, rich in mitochondria, accounting for approximately 40% of cardiac tissue volume [32-35]. Indeed, the impressive statistics that document the human heart beating ≈100000 times a day and cycling through up to 30 kg of ATP in this same period (the energy required to climb a ≈100-story building) vividly illustrate the importance of cardiac energy metabolism in both health and disease [33-36]. Specifically, as most cardiac energy production is derived from mitochondrial respiration, even a slight decline in mitochondrial function has the potential to cause significant problems in the energetic state of the heart [32]. In general, studies suggest that the impaired OXPHOS associated with HF is due to reduction in mitochondrial content, enzyme concentration, and enzymatic activity which are certainly important components of energy production [32, 37]. However, impaired OXPHOS could also be a result of substrate availability, altered structure of the mitochondria, reduced capacity of the PCr/ATP shuttle system, and the background metabolic state. In addition, HF is a complicated pathology, with multiple factors that can promote this condition (i.e. ischemic and non-ischemic etiology) and potentially result in varied mitochondrial deficits. Indeed, as HF-related mitochondrial changes are currently unclear, the purpose of the second study of this dissertation was to evaluate mitochondrial respiration, mitochondrial content, and free radical levels in cardiac muscle from HF, with a special interest in the potential differences between patients with Ischemic HF (iHF) and Non-ischemic HF (niHF). We tested two hypotheses: First, mitochondria in cardiac muscle from patients with iHF would have lower OXPHOS than that of patients with niHF. Second, the mitochondria in cardiac muscle from patients

with iHF would exhibit a greater uncoupled respiration, and greater free radical levels as well as attenuated OXPHOS efficiency in comparison to patients with niHF.

The role of the mitochondria in cardiac, skeletal, and vascular smooth muscle

The integrated function of cardiac, skeletal, and the smooth muscle of the vasculature is essential for O_2 delivery and utilization, especially, during exercise when synchronicity of these three muscle systems converge to help in determining functional capacity. The rhythmic contraction of cardiac muscle produces the driving force to convectively transport blood born O_2 to the periphery where skeletal muscle utilizes the O_2 for the metabolic requirements of locomotion [1, 38]. Blood vessels in the arterial system, relying on smooth muscle function, dictate the distribution of blood flow and O_2 transport, in response to O_2 demand [39, 40]. In comparison to cardiac and skeletal muscle, smooth muscle has distinct characteristics in terms of structure, responses to stimuli, electrical coupling between fibers, and speed of contraction. Although vascular smooth muscle contains mitochondria, which produce ATP through cellular respiration to meet the energy requirements of this tissue, little is known about the respiratory capacity and function of these mitochondria.

In cardiac muscle, mitochondria are the major metabolic organelles, taking up 35 % of a cells volume, and, at rest, producing almost 90% of ATP for cellular demands by β-oxidation [33-36]. However, heart mitochondria are metabolically very flexible organelles, sometimes described as ‘metabolic omnivores’, performing equally well with pyruvate, ketone, and lactate as fuel sources, dependent only on their availability [41].

Interestingly, cardiac muscle mitochondria are not only ATP producing ‘powerhouses’, but are also associated with cardio-protection against ischemia-reperfusion injury, cell signaling, and cell death [42]. Therefore, mitochondria in cardiac muscle have been intensively studied in heart diseases such as HF [41, 43, 44]. However, most of these studies are limited to animal or diseased human hearts as obtaining samples from healthy human hearts is difficult. Future studies utilizing samples from healthy human hearts are therefore warranted to investigate the comprehensive function and role of mitochondria across the complete health and disease spectrum.

In skeletal muscle, mitochondria are the primary controllers of cellular metabolism and signaling [45]. Typically 3-8% of skeletal muscle volume is mitochondria, with the variation being a consequence of skeletal muscle fiber type and adaptations to physical activity/inactivity [31, 46]. Mitochondrial ATP production is of great importance in skeletal muscle due to the high metabolic cost of locomotion and other forms of physical activity [47, 48]. Indeed, as indicated, skeletal muscle mitochondria adapt to different stimuli such as exercise and disease, resulting in a change in mitochondrial density and OXPHOS capacity [31, 46]. In fact, previous studies have suggested that a decline in skeletal muscle mitochondrial OXPHOS capacity and/or volume may contribute to muscle dysfunction [49-51]. Therefore, understanding and characterizing skeletal muscle mitochondrial function has significant implications in both health and disease.

Within blood vessels, both endothelial and smooth muscle cells contain mitochondria which produce ATP to maintain vascular tone, facilitate cellular transport, and power vascular cell secretion [15, 52]. These mitochondria typically comprise 3~5%

and ~5% of the smooth muscle and endothelial cell volume, respectively [53]. Recent evidence implicates vascular mitochondrial dysfunction in the development of vascular diseases [54-56]. Indeed, reductions in mitochondrial content and function have been linked to the age-related attenuation of vascular function [15], which warrants a comprehensive assessment of mitochondrial function in the blood vessels. As the comprehensive assessment of mitochondrial function in cardiac, skeletal, and smooth muscle has yet to be performed, the purpose of this third dissertation study was to determine the characteristics and respiratory function of mitochondria in cardiac, skeletal, and vascular smooth muscle. We tested two hypotheses: First, cardiac, skeletal, and vascular smooth muscles have distinct mitochondrial OXPHOS due to the different mitochondrial content. Second, intrinsic mitochondrial function would be different in cardiac, skeletal, and vascular smooth muscle.

The integration of the cardiac, skeletal, and vascular smooth muscle plays an essential role in O_2 transport and utilization. Therefore, a comprehensive understanding of these muscles, and the age- and disease-related alterations in both function and respiratory capacity, have important scientific and ultimately therapeutic implications. The overall purpose of this dissertation was to investigate the integrative function of the cardiac, skeletal, and vascular smooth muscle in terms of O_2 transport and utilization with aging and disease by: 1) investigating the effects of aging on vasodilatory capacity and kinetics in human SMFAs, 2) determining the impact of HF etiology on mitochondrial function, content, and free radical levels, and 3) comprehensively examining and comparing mitochondrial respiration in cardiac, skeletal, and smooth muscle.

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CHAPTER 2

The IMPACT OF AGE ON THE VASODILATORY FUNCTION OF HUMAN FEED ARTERIES

Abstract

Although advancing age is associated with attenuated skeletal muscle blood flow, and skeletal muscle feed arteries (SMFAs) have been recognized to play a regulatory role in the vasculature, little is known about the impact of age on the vasodilatory capacity of human SMFAs. Therefore, endothelium-dependent and -independent vasodilation was assessed in SMFAs (diameter 544±63 μm) obtained from 24, equally represented, young (33±2 yr) and old (71±2 yr) subjects in response to three stimuli 1) flow-induced shear stress, 2) acetylcholine (ACh), and 3) sodium nitropusside (SNP). Both assessments of endothelium-dependent vasodilation, flow (Young: 68±1; Old: 32±7 %) and ACh (Young: 92±3; Old: 73±4 %), were significantly blunted (p<0.05) in the SMFAs of the old compared to the young, with no such age-related differences in endothelium-independent vasodilation (SNP). Furthermore, in response to an increase in flow-induced shear stress, vasodilation kinetics (Tau: Young: 55±1; Old: 92±7 s), and eNOS activation (Phospho-eNOS^{s1177}/total eNOS, Young: 1.0 ± 0.1; Old: 0.2±0.1) were also significantly attenuated in the old compared to the young (p<0.05). These findings reveal that the endothelium-dependent vasodilatory capacity, including vasodilation kinetics, but not smooth muscle function, of human SMFAs is blunted with age. Given the regulatory role of SMFAs in skeletal muscle blood flow, these findings may explain, at least in part, the attenuated perfusion of skeletal muscle which likely contributes to exercise intolerance in the elderly

Introduction

Attenuated cardiovascular function, as characterized by impaired oxygen delivery to skeletal muscle, is a well-documented phenomenon associated with aging (23, 30, 34)

and is likely responsible, at least in part, for the diminished exercise capacity in the elderly. Certainly, with advancing age, due to a decrease in both maximum heart rate and stroke volume, maximum cardiac output is compromised (10, 30, 43). Interestingly, not as a direct consequence, but in addition to this attenuated central cardiovascular response, many studies have also documented attenuated leg blood flow and vascular conductance during exercise in the old compared to the young (24, 28, 33, 35, 42). However, the exact location and mechanisms responsible for this diminished peripheral blood flow response is currently not well understood.

In animals, it has been well documented that SMFAs, the inlets to the muscle bed upstream of the arterioles, due to location and vasoactive capacity, are primary blood flow regulators during physical activity (37, 44). Furthermore, additional animal work suggests that aging impairs endothelium-dependent dilation in SMFAs (45). Building upon these animal studies, Ives et al. (17, 19) recently translated these observations, using a pharmacological approach, providing evidence that human SMFAs also have the potential to regulate skeletal muscle perfusion. However, although the likely importance of human SMFAs has been highlighted, few such studies have been performed because these vessels are difficult to obtain and therefore little is currently known about the impact of age on SMFA vasomotor function.

Utilizing *in vitro* methods to study animal skeletal muscle arterioles, which are downstream to the SMFAs, Muller-Delp et al. (29, 30) documented that local muscle blood flow was reduced in old rats, and proposed that the mechanism responsible for this age-related attenuation was impaired endothelium-dependent vasodilation. Additionally, Behnke et al. (2) recently revealed that the rate of skeletal muscle arteriole vasodilation,

likely reflective of the kinetics of muscle perfusion within the muscle, is blunted in old compared with young mice. Therefore, in terms of the age-related reduction in oxygen delivery to skeletal muscle, these and other studies have identified a role for diminished nitric oxide (NO) bioavailability and, subsequently, endothelial-mediated vasodilatory capacity in skeletal muscle resistance arteries and arterioles in animal models (8, 14, 30). However, whether vascular dysfunction associated with advancing age, at the level of the SMFA in humans, contributes to the diminished peripheral blood flow associated with the elderly remains to be determined.

Consequently, utilizing pressure myography, this study sought to further examine the vasomotor function of human SMFAs with a specific focus on the impact of age. We tested three hypotheses: First, supporting the concept that SMFAs have regulatory potential, both young and old human SMFAs will exhibit significant vasodilatory capacity when stimulated by flow-induced shear stress, acetylcholine (ACh), and sodium nitroprusside (SNP). Second, the endothelium-mediated vasodilatory response, stimulated by flow-induced shear stress and ACh, will be attenuated in the old compared to the young, whereas the endothelium-independent vasodilatory response (SNP) will not be affected by age. Third, likely due to impaired endothelium-dependent vasodilation, the kinetics of flow-induced vasodilation will be slower in the old compared to young. If these hypotheses are proven to be correct, these findings will add to the understanding of skeletal muscle blood flow regulation with advancing age and have implications for the targeting of interventions aimed at maintaining physical function in the ever growing elderly population.

Methods

Subjects and general procedures

A total of 40 SMFAs from the axillary and inguinal regions were obtained from young (~33 yrs, n=20) and old (~71 yrs, n=20) subjects during melanoma-related surgeries. Twenty-four, equally represented, young and old SMFAs were used in all protocols except the Western blot analyses following only flow exposure which were performed in the 16 additional SMFAs (8 young and 8 old). All subjects were free from cancer and chemotherapy, but there were no other specific exclusion criteria for this study, although all medical conditions and medications were noted. All protocols were approved by the Institutional Review Board of the University of Utah and Salt Lake City VA Medical Center, carried out in accordance with the Declaration of Helsinki, and written informed consent was obtained from all subjects prior to surgery.

Vessel harvest and preparation

SMFAs (outer diameter ~500 μm, length 2-3.0 mm) from the axillary (e.g. serratus anterior or latissimus dorsi muscles) and inguinal (e.g. hip adductors or quadriceps femoris muscles) regions, obtained during sentinel node biopsy for melanoma surgery at the Huntsman Cancer Hospital and the Salt Lake City VA Medical Center, were studied. Patients were anaesthetized using a general protocol: propofol, fentanyl, benzodiazepines, and succinylcholine (32). SMFAs were harvested after dissecting out sentinel lymph nodes for clinical analysis and were identified and classified based upon being a vascular inlet into a muscle bed, structure, coloration, and pulsatile bleed pattern

(18). SMFAs were ligated, excised, and immediately placed in iced normal physiological saline solution (PSS) prior to transfer to the laboratory within 15 min of harvesting (19).

Vessel function protocols

SMFAs from 24, equally represented, young and old subjects were assessed in these protocols. Initially, perivascular adipose and/or connective tissue around SMFAs was removed under a dissecting microscope (SZX10; Olympus, Center Valley, PA, USA) in cold (4 ◦C) PSS containing (mM): 145.0 NaCl, 4.7 KCL, 2.0 $CaCl_2$, 1.17 $MgSO_4$, 5.0 glucose, 2.0 pyruvate, 0.02 EDTA, 3.0 MOPS buffer, and 1 g $(100mL)^{-1}$ BSA at pH 7.4. Function of SMFAs was measured using pressure myography organ baths (110p; DMT Systems, Aarhus, Denmark). The arteries were cannulated at both ends with micropipette tips and the vessel outer diameters were recorded under an inverted microscope with a video camera (TS100; Nikon Eclipse, Melville, NY, USA), with data streamed in real time to edge detection software (DMT VAS v.2.0), monitored at a sampling rate of 1 kHz. Fluid leak was detected by pressurizing the vessel, with intraluminal pressure set at 60 mmHg, closing the cannulas to the fluid reservoirs and assessing the capacity to maintain vessel diameter. Arteries free from leaks were then warmed to 37°C, allowed to develop spontaneous tone for a 30 min equilibration period (19), and then underwent vasodilation assessments and, ultimately, Western blot analysis.

Vasodilation assessments

Vasodilation (%) was assessed in response to three stimuli: First, to assess the endothelium-mediated vasodilatory response to flow-induced shear stress, intraluminal

flow was developed. This was achieved by altering the heights of the independent fluid reservoirs, contiguous with the SMFAs, in equal and opposite directions so that a pressure difference was developed across the vessel without altering mean intraluminal pressure (2). Pilot work revealed a large increase in % vasodilation in response to the flow induced by a pressure difference of 30 mmHg compared to 15 mmHg and more subtle increases in % vasodilation from 30 to 44 mmHg and 44 to 60 mmHg. Hence, a single pressure difference of 30 mmHg, which yielded an approximate flow rate of 30 ul/min, was adopted for all subsequent flow experiments. With this flow rate and typical SMFA internal diameter, an expected shear rate of ~500 s^{-1} was calculated using the following equation: 8×mean velocity/vessel diameter (31). Second, to assess endothelium-dependent vasodilation pharmacologically, an ACh dose response curve (ACh, 10^{-7} to 10^{-3} M) was performed following pre-constriction with PE (10^{-6} to 10^{-4} M) to ~70% of the maximum PE response. Third, to assess endothelium-independent vasodilation, a SNP dose response curve was performed (10^{-9} to 10^{-4}M) following pre-constriction with PE (10^{-6} to 10^{-4} M) to ~70% of the maximum PE response. Vasodilation sensitivity was defined as the concentration of ACh or SNP that elicited 50% of the maximal response (EC_{50}) which was calculated by a sigmoidal parameter, as described previously (18). Calcium free normal physiological saline solution was used to measure maximum passive relaxation of the feed arteries.

Western blot analysis

Baseline protein expression of phosphorylated eNOS at Serine ^{1177}(p-eNOS ^{ser1177}, catalog no. 9570, Cell Signaling, Boston, MA), a well-described eNOS activation site (9),

and total eNOS (catalog no. 610296, BD Transduction, San Jose, CA) analysis was performed (47), using a subset (8 Young and 8 Old) of unused pieces of the same arteries which were assessed for vessel function. An additional 16 (8 Young and 8 Old) arteries were snap frozen in liquid nitrogen immediately after being exposed to only 6 min of flow at ~30 ± 1 ul/min to measure the impact of flow on eNOS and p-eNOS ^{ser1177} protein expression. GAPDH (ab9485, Abcam, Cambridge, MA) was utilized as a loading control and therefore the protein data from the Western blot analyses were normalized GAPDH. The Western blots were performed in duplicate and the data averaged.

Free radical measurement

A subset (8 Young and 8 Old) of unused pieces of the same arteries which were assessed for vessel function were used in this protocol. Electron paramagnetic resonance (EPR) spectroscopy was performed on frozen tissue to directly assess free radical concentration, utilizing the superoxide specific spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH) (Enzo life sciences, Farmingdale, NY,USA), as previously described (4). An EPR spectrometer (EMX X band, Bruker, Manning Park Billerica, MA) was utilized in combination with commercially available software (version 2.11, Bruker Win EPR System) to calculate the area under the curve of the EPR spectroscopy signal by double integration.

Calculations

Percent vasodilation was used for data expression to account for baseline differences in vessel diameter, and calculated using the following equation:

$$(DT-Dp/Di-Dp)\times 100$$

where Dt is the recorded diameter at a given time point, Dp is the diameter recorded after the addition of the vasoactive agent (i.e. pre-constriction diameter), and Di is the diameter recorded immediately before the addition of the vasoactive agent (initial diameter) (2).

Vasodilation kinetics were determined by fitting the time-dependent vessel diameter changes during the shear stimulus period to a single exponential curve described by the following equation:

$$Diameter_t = Diameter_{base} + Diameter_{end}(1-e^{-(t-TD/\ tau)})$$

where $Diameter_t$ is the change in diameter at time *t*, $Diameter_{base}$ is the baseline diameter and $Diameter_{end}$ refers to the maximal vessel diameter in response to the shear stimulus, TD is the time delay before the onset of diameter change, and tau is the time constant to reach 63% of the amplitude of the response (2).

Statistical analyses

Statistical analyses were performed using SPSS statistical software (SPSS version 17. SPSS, Chicago Il, USA). Group differences and vessel characteristics were compared using 2 way ANOVA. One-way repeated measures ANOVA were used to determine significant changes in vessel diameter for dose-responses to ACh and SNP. A student *t*- test was performed to identify the alteration in vessel diameter if the ANOVA was significant. For all analyses, a p-value of < 0.05 was considered significantly different. All data are expressed as mean ± SEM.

Results

Subject characteristics

A total of 40 subjects participated in this study, with 24 SMFAs harvested and assessed for vasodilatory function and basal eNOS and p-eNOS ^{ser1177} protein expression and superoxide levels assessed in two subsets of 16 of these 24 vessels; Western blot analyses, following only flow exposure, was performed in the 16 additional SMFAs. In all assessments, the young and old were equally represented. The subject characteristics, obtained from preoperative examination medical records, are presented in Table 2.1. Note that users of cardiovascular (β-blocker, angiotensin- converting enzyme inhibitor, diuretic, Ca^{2+} channel blocker, etc.), diabetic (insulin, metformin, etc.), and cancer-related medications were excluded from the study. Also, it should be noted that all the blood chemistry and complete blood count results (Table 2.1) were within normal range, suggesting that the subjects who participated in this study were relatively healthy.

Vessel characteristics

SMFAs were harvested from either the inguinal (n=27) and axial (n=13) regions and, in line with our previous observations (18, 19), there were no statistical differences in vasoconstriction or vasorelaxation responses in terms of anatomic origin or sex. Basal, unpressurized, outer diameter of the SMFAs was not statistically different between young and old (Young: 510 ± 12 *u*m; Old: 514 ± 15 *u*m). Additionally, maximal outer diameter of the SMFAs, achieved by Ca^{2+} free NPSS incubation, in young and old was not statistically different (Young: 758 ± 19 *u*m; Old 752 ± 14 *u*m).

Vasodilatory response to intraluminal flow

The PE-induced pre-constriction of the SMFAs prior to the flow stimulus was similar between groups (Old: 68 ± 5; Young 69 ± 4 %, $P > 0.05$). The greatest vasodilation in response to the intraluminal flow of 30 ± 1 ul/min was significantly reduced in the old compared to young (Old: 32 ± 7; Young 68 ± 1 %, $P < 0.05$) (Figures 2.1 and 2.2). The Tau, an indicator of vasodilation kinetics, for the flow-induced vasodilation was significantly slower in the old compared to young (Old: 92 ± 7; Young: 55 ± 13 s, $P < 0.05$). Additionally, the time delay before a statistically significant flow-induced change in diameter occurred tended to be slower in the old compared to young (Old: 8 ± 2; Young: 3 ± 1s), but did not achieve statistical significance (p=0.07)(Figure 2.2).

Vasodilatory response to Ach and SNP

The PE-induced pre-constriction of the SMFAs prior to the both the ACh and SNP vasodilatory stimuli were similar between groups (Old: 68 ± 5; Young 69 ± 5 %, $P > 0.05$). The SMFAs from the old exhibited significantly attenuated vasodilation in response to the highest dose of ACh (10^{-3} M), in the ACh dose response curve, than the young (Old: 73 ± 4; Young: 92 ± 3%, $P < 0.05$) (Figure 2.1 and 2.2A). In contrast, the greatest vasodilatory response to SNP (10^{-4} M), in the SNP dose response curve, was similar between old and young (Old: 112 ± 11 %; Young: 102 ± 4 %, $P > 0.05$) (Figures 2.1 and 2.2B) . The sensitivity to ACh, assessed by log EC_{50}, was significantly attenuated in the old compared to young (Old: -5.5 ± 0.3; Young: -4.1 ± 0.4, $P < 0.05$), whereas the

sensitivity to SNP was similar between old and young (Old: -5.75 ± 0.5; Young: -5.77 ± 0.5, $P > 0.05$)(Figure 2.3).

Protein expression

The baseline ratio of p-eNOS ^{ser1177}/total eNOS was significantly greater in old compared to young (Old: 2.8 ± 0.08; Young: 1.0 ± 0.04 fold change, relative to young, $P < 0.05$) (Figure 2.4A). However, p-eNOS ^{ser1177}, an activation site of eNOS on the Serine ^{1177} residue, to total eNOS protein expression in response to a 6 min flow stimulus was significantly lower in old compared to young (Old: 0.2 ± 0.08; Young: 1.0 ± 0.04 fold change, relative to young, $P < 0.05$) (Figure 2.4B).

Free radicals

The baseline EPR spectrospcopy signal for the CMH adduct, an index of superoxide concentration, was greater in old compared to young (Old: 3.9 ± 1.0: Young: 1.7 ± 0.1 AUC / mg, P<0.05)(Figure 2.5).

Discussion

Although aging is associated with attenuated skeletal muscle blood flow and SMFAs have been recognized to play a regulatory role in the vasculature, little is known about the impact age on the vasodilatory capacity of human SMFAs. In this regard, there are several interesting and novel findings of this study. First, these data both confirm and extend the inference that human SMFAs are likely regulatory in nature. Specifically, the SMFAs are capable of significantly increasing vessel diameter in response to both

increased shear stress and pharmacological vasodilators, the magnitude of which, *in vivo*, would likely result in significant changes in vascular conductance. Second, the magnitude of the SMFAs endothelium-mediated vasodilatory capacity, assessed by the vasodilatory response to shear stress and ACh, is significantly attenuated with advancing age. However, in contrast, aging did not impact smooth muscle function. Third, in agreement with an age-related decrement in endothelium-mediated vascular function, shear-induced SMFA vasodilation kinetics are significantly attenuated with age. Finally, providing mechanistic insight into the age-related attenuation in SMFA vasodilatory function, shear-induced eNOS phosphorylation is significantly reduced with age, suggestive of a role for NO bioavailability in these findings. Given the potential impact of SMFAs in the regulation of blood flow during exercise, these findings may explain, at least in part, the attenuated perfusion of skeletal muscle with advancing age, which likely contributes to exercise intolerance in the elderly.

Implication of attenuated SMFA vasodilation and vasodilation kinetics with age

The regulation of oxygen delivery and therefore blood flow at rest and during exercise is an important component of homeostasis in both humans and animals (6, 19). In animals, it has been well documented that, in comparison to their younger counterparts, older animals exhibit an attenuated maximum vasodilatory capacity in skeletal muscle arterioles, which results in an impaired oxygen delivery to working muscles (29, 30). Furthermore, Behnke et al. (2, 3), using an isolated vessel approach, revealed an age-related impairment in vasodilation kinetics. This would likely result in a temporal mismatch between oxygen delivery and oxygen consumption in skeletal muscle during

the rest to exercise transition, ultimately resulting in a low microvascular partial pressure of oxygen with advancing age. Although this age-related transient attenuation in microvascular oxygen availability has been documented to be a consequence of delayed blood flow to the muscle at the onset of contraction in animals (2), to our knowledge, until now, there had not even been a single study that examined the effect of age on human SMFA vasodilation kinetics, which may have the same downstream consequences. Indeed, this appears to be the first study demonstrating a reduced magnitude of vasodilation and vasodilation kinetics in human SMFAs as consequence of age (Figures 2.1, 2.2, 2.3, and Table 2.1) and the impact on muscle oxygen availability still needs to be determined.

Interestingly, at the onset of flow, the vasodilatory response of the current SMFAs, specifically the time to reach the peak diameter, was similar to previous studies examining other human vessels (5, 27), but significantly slower (~200 s) compared to the animal study performed by Behnke et al. (8-23 s) (2). The tau was also significantly slower in the human SMFAs compared to the animal arterioles (2). However, both the slower tau and the time to reach the peak shear-induced vasodilatory response in the human SMFAs may be due to the larger size of these vessels compared to the arterioles of much smaller animals (31). Another clear distinction between these vessels is location, with the SMFAs being external to the muscle while arterioles are internal. Thus, although both vessel types may have similar roles in terms of blood flow regulation, each may exhibit differing vasodilatory characteristics simply due to being a part of differing vascular beds (31). Of note, however, and in agreement with our previous studies (19), but certainly not a focus of the current work, there was no evidence of different SMFA

vasoactive responses as a consequence of anatomical location (i.e. axillary and inguinal regions). This implies that SMFAs from upper and lower limbs perform similarly in terms of vasodilation.

Although it is currently not possible to quantify the exact physiological shear forces experienced by SMFA in humans, based upon studies in animals, Lipowsky et al. (25) suggested that an acceptable physiological shear rate for human arterioles would be in the 250-1500 s^{-1} range. Furthermore, Fisslthaler et al. (13) demonstrated that the induction of a shear stress of 12 dynes cm^{-2}, equivalent to shear rate of 200 s^{-1}, can significantly increase eNOS activation in cultured human umbilical vein endothelial cells. It should be noted that procedures in the current study resulted in a shear rate of ~500 s^{-1}, which stimulated significant SMFA dilation. Interestingly, the delta pressure of ~30 mmHg, employed in the current study, yielded a significantly greater vasodilatory response than the delta pressure of ~15 mmHg, while greater increases in pressure difference (Δ~45 and Δ ~60 mmHg), assessed in pilot studies, resulted in more subtle increases in vasodilation. These data could be interpreted to indicate that a shear rate of ~500 s^{-1} is a physiologically relevant stimulus for human SMFAs, and advancing age blunts the vasodilatory response to such a level of shear.

Potential mechanisms responsible for the age-related attenuation in SMFA vasodilation

Previous animal studies have suggested that there is an attenuated vascular conductance and a limited increase in red blood cell flux over time in muscle during dynamic exercise with advancing age (7, 15), indicative of compromised peripheral circulatory function (2, 7, 16). These prior studies, and other work focused upon the

impact of age on the microvasculature during exercise in animals, have revealed slower endothelium-dependent vasodilator dynamics in skeletal muscle arterioles, due to the reduced NO bioavailability in the old (2, 16, 30). In agreement with these studies, the current results extend these findings to human SMFAs, revealing that aging reduces flow-induced (Figures 2.1 and 2.2) and ACh-induced (Figure 2.3) vasodilation, both indicators of endothelium-dependent dysfunction.

Furthermore, this study revealed an attenuated increase in p-eNOS ^{ser1177} to total eNOS protein expression, a well-described activation site on eNOS (41), in the SMFAs from the old subjects in response to an increase in shear stress, providing a mechanistic basis for the age-related attenuation in both the magnitude of vasodilation and vasodilation kinetics (Figure 2.4B). Interestingly, SMFAs from the old subjects also exhibited a significantly greater basal p-eNOS ^{ser1177}/Total eNOS protein expression compared to the young (Figure 2.4A) which contrasts with the predominant findings in the microvasculature of animals (40, 41, 47). However, these findings are well aligned with previous assessments of the vasculature in humans that have documented a greater activation of p-eNOS ^{ser1177} with age at rest which was interpreted as an attempt to compensate for low NO bioavailibity (11, 36). Overall, these findings suggest that baseline eNOS activation is increased in the SMFAs of the old to compensate for the attenuated NO bioavailability; however, the SMFAs from the old appear less capable of activating eNOS in response to an increased shear stimulus compared to the young. Additionally, the EC_{50} for ACh, indicative of the sensitivity of muscarinic receptors in the endothelium, was reduced with age which may also play a role in the attenuated vasodilation kinetics in old.

It has been suggested that an increase in the production of reactive oxygen species (ROS) and/or limited antioxidant capacity with advancing age attenuates endothelial function in humans and animals (1, 30, 46). Interestingly, albeit in the basal state, the current study provides evidence of greater superoxide production in the SMFAs of the old compared to the young, as directly assessed by EPR spectroscopy (Figure 2.5). The reaction of NO with superoxide, to produce peroxynitrite (36), plays a major role in lowering NO bioavailibity, not only directly, but indirectly as peroxynitrite can uncouple eNOS. Indeed, Liu et al. (27) have documented that shear stress-induced ROS production in human coronary arteries decreases NO bioavailability, and others have documented that ROS impair NO-mediated vasodilation in the peripheral arteries with age (12, 26, 27, 29). Furthermore, recent studies suggest that in the presence of blunted NO-mediated dilation, due to decreased NO bioavailability, compensatory vasodilatory mechanisms (i.e. H_2O_2) are favored in both old and diseased arteries (5, 22). This may be another potential explanation for the attenuated magnitude of flow-mediated vasodilation and the slowed vasodilation kinetics in the SMFAs of the old subjects. This potential compensatory mechanism, H_2O_2-mediated vasodilation, may not be as effective as NO in terms of either vasodilatory capacity or the speed of vasodilation; however, such speculation certainly needs further studies to confirm this contention. Regardless, the current study confirms that aging attenuates human SMFA vasodilatory capacity as well as vasodilation kinetics of human SMFAs and provides evidence that, mechanistically, this is likely due to decreased NO bioavailability.

The regulatory function of SMFAs

Previous animal studies have suggested that SMFAs are capable of altering muscle blood flow by manipulating vascular conductance in accordance with metabolic requirements (21, 38). This vasoconstriction and vasodilation, previously documented *in vitro*, would likely translate into significant alterations in basal vascular resistance *in vivo* (37-39). Recent work by our group has provided translational evidence of the regulatory capability of human SMFAs, elicited by pharmacological stimulation, by documenting significant changes in calculated vascular conductance, (19). In addition to these previous studies, and similar to that observed in a rodent model (20), the current study confirms and extends this evidence of the regulatory potential of human SMFAs by documenting vasodilation in response to a more physiologically relevant stimulus, an increase in shear stress. These findings suggest that SMFAs may be an important factor in regulating local blood flow at rest and during challenges to homeostasis such as exercise and orthostasis in humans. Importantly, this regulatory potential appears to be compromised with advancing age and this may have significant consequences in terms of skeletal muscle blood flow in the elderly.

Summary

This study utilized both a physiologically relevant flow-induced increase in shear stress and a pharmacologically-induced vasodilation to assess the impact of aging on vasodilatory capacity and vasodilation kinetics in human SMFAs. It was determined that the endothelium-dependent vasodilatory capacity and vasodilation kinetics, but not endothelial-independent smooth muscle function, of human SMFAs is blunted with age.

This attenuated endothelial-dependent vascular function seems to be explained by attenuated eNOS activation and subsequently NO bioavailability, and may be a consequence of elevated ROS production with age. Given the likely regulatory role of human SMFAs in skeletal muscle blood flow, these findings may explain, at least in part, the attenuated perfusion of skeletal muscle with advancing age which likely contributes to exercise intolerance in the elderly.

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Table 2.1. Subject characteristics

	Young (n=20)	Old (n=20)
Age (year)	33±2	71±2 *
Male (male/total n)	17/20	18/20
Female (female/total n)	3/20	2/20
Height (cm)	174±2	169±9
Body mass (kg)	79±10	72±8
BMI (kg m^{-2})	26±4	25±5
Systolic blood pressure (mmHg)	118.2±4.4	120.2±4.8
Diastolic blood pressure (mmHg)	75.1±2.0	81.1±2.0
Glucose (mg dl^{-1})	108.1±8.4	109.3±6.8
Blood urea nitrogen (mg dl^{-1})	17.4±2.0	16.9±3.0
Creatinine (mg dl^{-1})	0.8±0.2	0.9±0.1
Albumin (g dl^{-1})	4.2±0.4	4.3±0.5
Lactate dehydrogenase (U L^{-1})	501.4±34.3	504.3±36.1
Hemoglobin (g dl^{-1})	14.4±0.9	13.5±0.8
White blood Cells (thousands per microliter, K ul^{-1})	5.2±0.8	8.4±0.9
Red blood Cells (millions per microliter, M ul^{-1})	4.5±0.3	4.2±0.2
Platelets (K ul^{-1})	246.2±20.4	254±30.5
Hematocrit (%)	42.8±1.9	40.3±1.3
Lymphocytes (%)	30.4±4.0	32.4±2.0
Monocytes (%)	8.0±1.6	7.8±1.3
Medications (Users/n)		
Diuretics	0/12	0/12
Angiotensin- converting enzyme inhibitors	0/12	0/12
Diabetic drugs	0/12	0/12
Statins	0/12	2/12

Data are expressed as mean ± SE or number of subjects (out of the total number). * significantly different from the young, $P < 0.05$

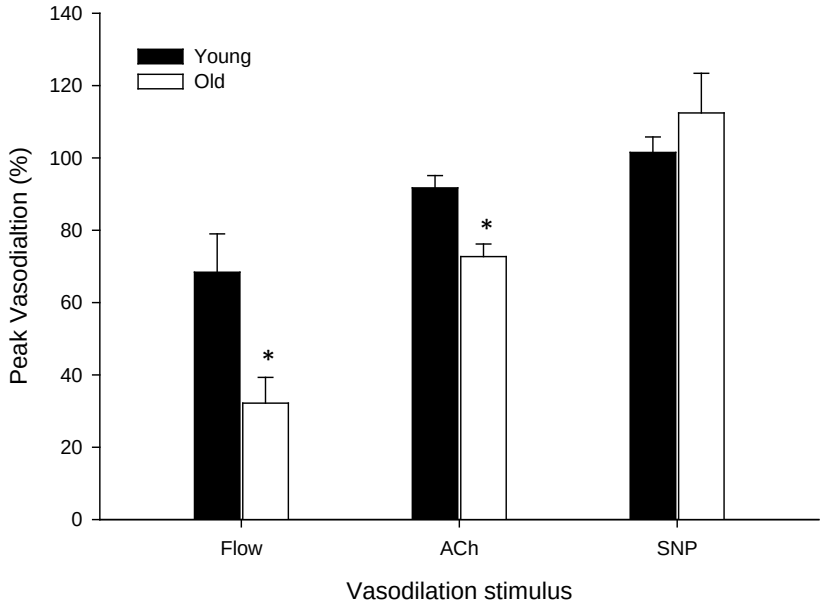

Figure 2.1. Skeletal muscle feed artery peak % vasodilation induced by flow, acetylcholine (ACh), and sodium nitropruside (SNP) in young and old subjects. n = 12 young and 12 old. Data are expressed as mean ± SE. * significantly different from the young, $P < 0.05$.

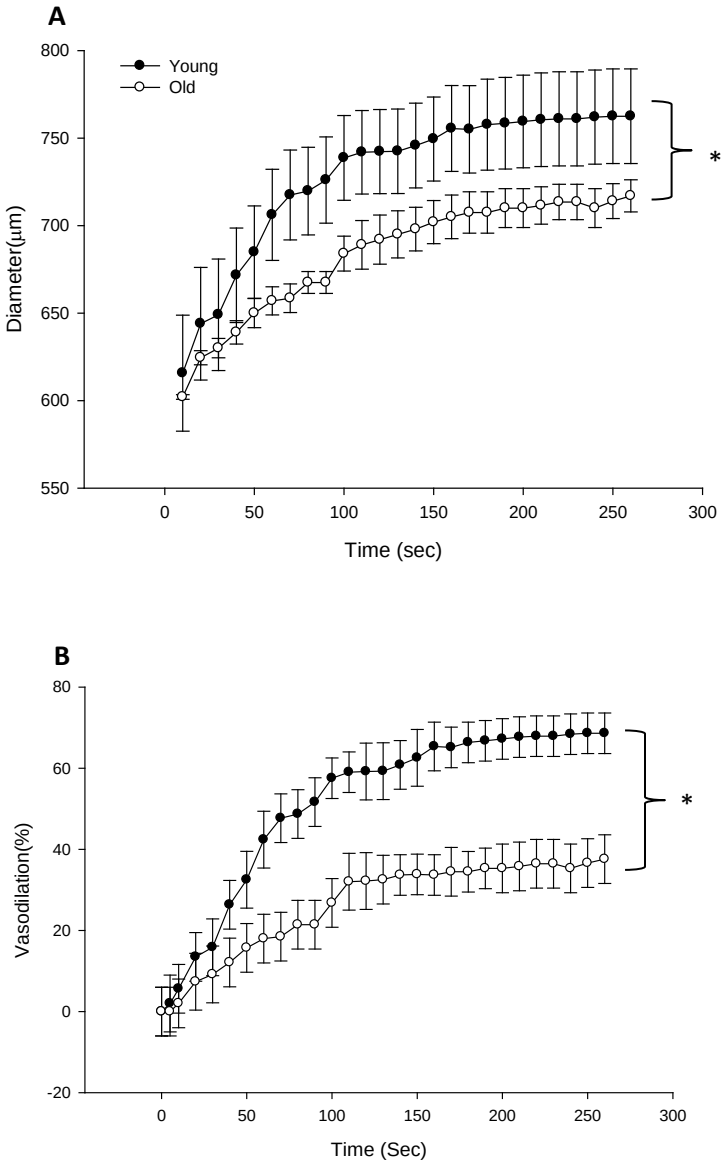

Figure 2.2. Skeletal muscle feed artery vasodilatation kinetics illustrated as absolute change in diameter (A) and % vasodilation (B) in young and old subjects. n = 12 young and 12 old. Data are expressed as mean ± SE. * significant difference between the young and old, $P < 0.05$.

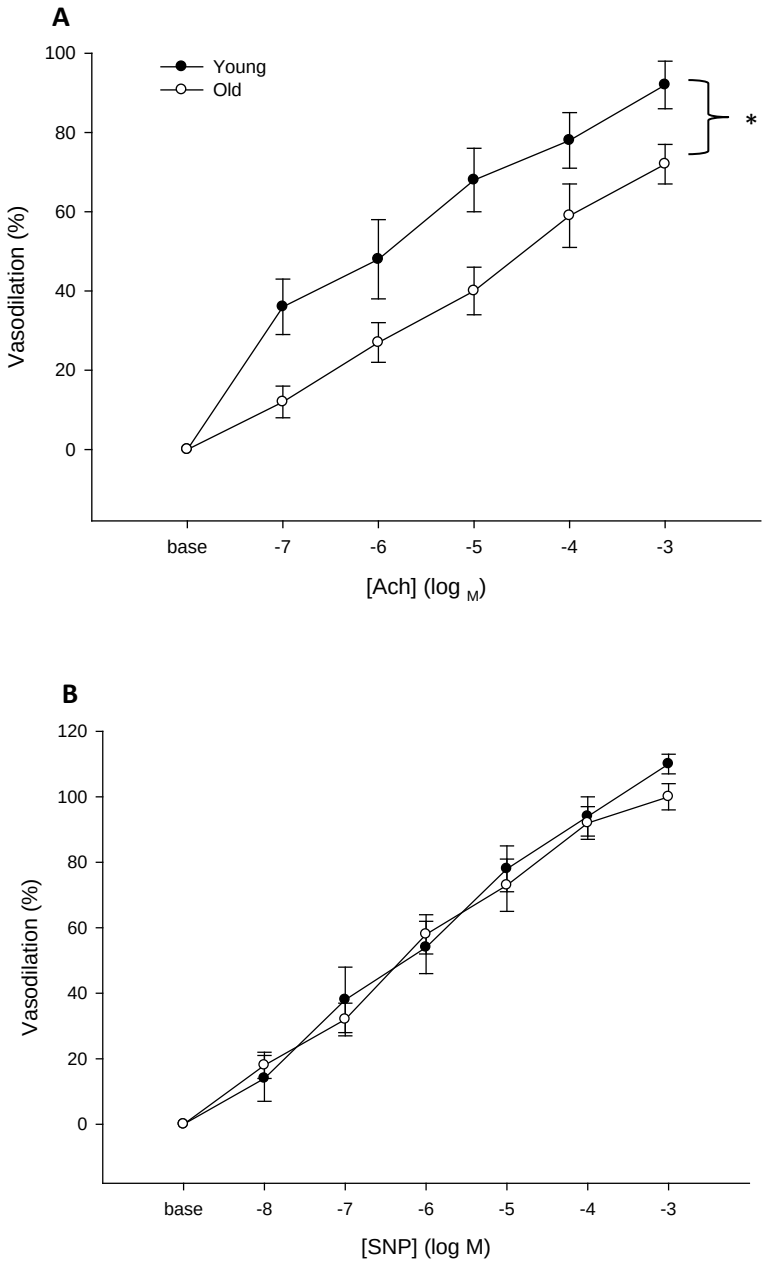

Figure 2.3. Skeletal muscle feed artery endothelial-dependent vasodilation, induced by increasing doses of acetylcholine (ACh) (A), and endothelial-independent vasodilation, induced by increasing doses of sodium nitropruside (SNP) (B) in young and old subjects. n = 12 young and 12 old. Data are expressed as mean ± SE. * significant difference between young and old, $P < 0.05$.

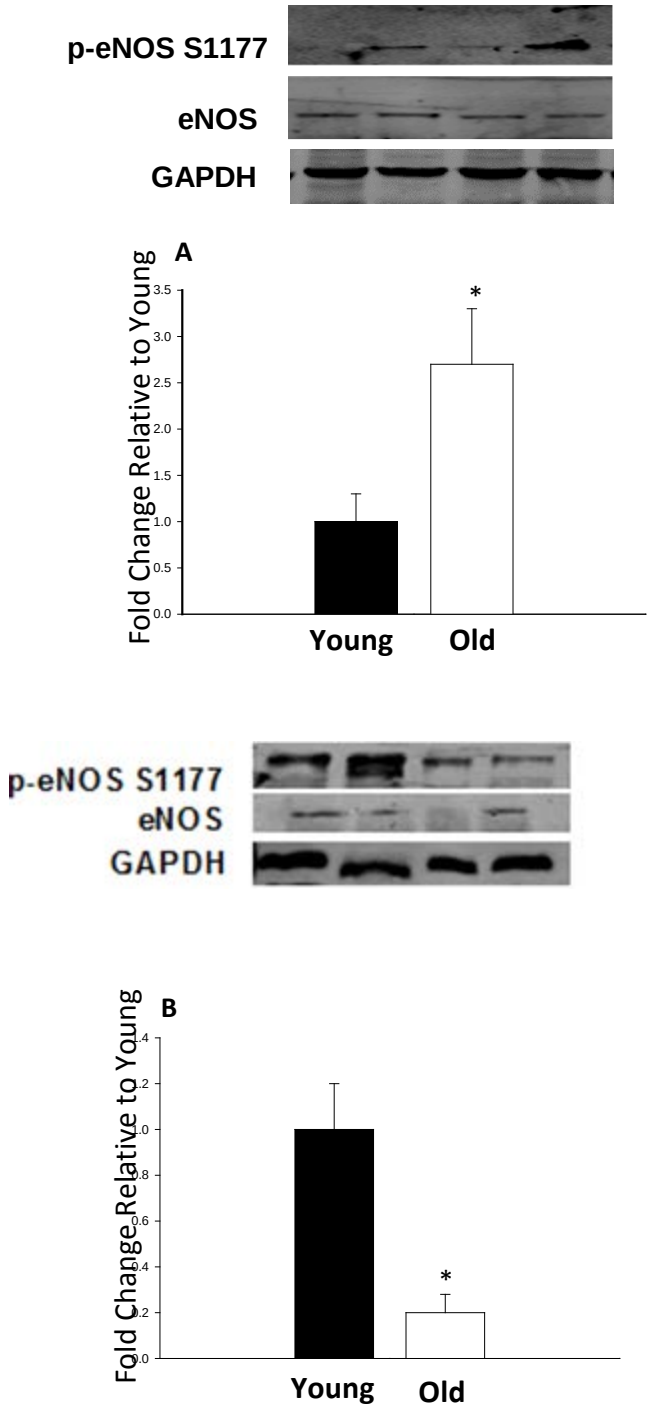

Figure 2.4. Total eNOS and phosphorylated endothelial nitric oxide at Serine ^{1177} (p-eNOS ^{ser1177}) in skeletal muscle feed arteries of young and old subjects in basal conditions (A) and the change induced by 6 min of flow inducing a shear rate of ~500 s^{-1} (B). n = 8 young and 8 old in each panel. Data are expressed as mean ± SE. * significantly different from the young, $P < 0.05$.

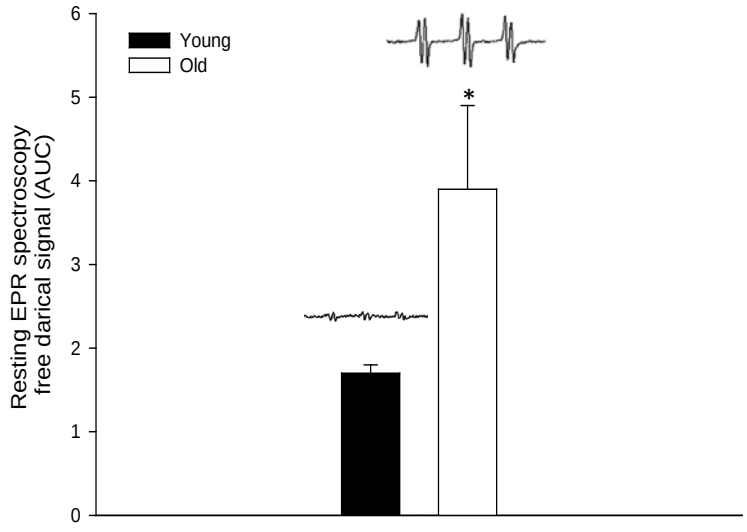

Figure 2.5. Basal superoxide level in young and old skeletal muscle feed arteries assessed by electron paramagnetic resonance (EPR) spectroscopy. EPR signal expressed as the area under the curve (AUC, arbitrary units). Representative spectroscopy signals inlayed. n = 8 young and 8 old. Data are expressed as mean ± SE. * significantly different from the young, $P < 0.05$

CHAPTER 3

MITOCHONDRIAL FUNCTION IN HEART FAILURE: THE IMPACT OF ISCHEMIC AND NON-ISCHEMIC ETIOLOGY

Abstract

Although cardiac mitochondrial dysfunction is associated with heart failure (HF), this is a complex syndrome with two predominant etiologies, ischemic HF (iHF) and non-ischemic HF (niHF), and the impact of mitochondrial dysfunction in these two distinct forms of HF is unknown. To determine the impact of HF etiology on mitochondrial function, respiration was measured in permeabilized cardiac muscle fibers from patients with iHF, niHF, and healthy donor hearts (HdH). Oxidative phosphorylation capacity (OXPHOS), assessed as Complex I, II, and I+II state 3 respiration, fell progressively from HdH to niHF, to iHF (Complex I+II: 54±1; 34±4; 27±3 pmol·s^{-1}·mg^{-1}) as did citrate synthase activity (CSA) (206±18; 129±6; 82±6 *u*mol·g^{-1}·min^{-1}). Although still significantly lower than HdH, normalization of OXPHOS by CSA negated the difference in mass specific OXPHOS between iHF and niHF. Interestingly, Complex I state 2 respiration increased progressively from HdH, to niHF, to iHF, whether or not normalized for CSA (23.2±3; 11±3; 6±2 pmol·mg^{-1}·CSA), such that the respiratory control ratio (RCR) fell in the same manner across groups (5.3±0.2; 2.4±0.3; 1.3±0.2, $P<0.05$). Finally, both the total free radical concentration (60± 6; 46 ± 4 AU) and level of mitochondrial derived superoxide (1.0±0.2; 0.7±0.1 AU) were greater in iHF compared to niHF, respectively. Thus, the HF-related attenuation in OXPHOS actually appears to be independent of etiology when the lower mitochondrial content of iHF is taken into account. However, it is evidence of deleterious intrinsic mitochondrial changes in iHF, compared to niHF, including greater proton leak, attenuated OXPHOS efficiency, and greater free radical levels.

Introduction

The heart is a vital organ with a high metabolic demand and is subsequently rich in mitochondria, with these energy producing organelles accounting for approximately 35% of cardiac tissue volume (48) and generating up to 90% of the heart's requirement for ATP (23, 31, 35). Due to an impaired energetic state, as evidenced by a reduction in the phosphocreatine to ATP ratio, the heart in HF has been described as an 'engine without fuel' and mitochondrial dysfunction has been implicated as an important component of this disease (29, 43). Such an attenuation in cardiac myocyte energy production may be the result of an alteration in the ratio of coupled, ATP producing, to uncoupled respiration, not ATP producing (2), potentially influencing the contractile capacity of the heart by creating a mismatch between energy supply and demand (48). However, previous studies focused upon HF and mitochondrial function have reported inconsistent results in terms of HF-related cellular adaptations including cell structure, mitochondrial substrate metabolism, and mitochondrial enzymes (11, 14, 32). These varied findings may, at least in part, be due to the differing impact of the multiple common co-morbidities associated with HF such as insulin resistance, diabetes, and the subsequently elevated lipid accumulation in cardiac tissue (7, 15)

It is apparent that the exact impact of cardiomyopathy on mitochondrial function is not well characterized and may actually be further complicated by the fact that HF is a complex syndrome with two predominant etiologies, iHF and niHF. The first and most common, iHF, is coronary artery disease (CAD)-dependent, while niHF is not related to CAD and includes dilated cardiomyopathy, hypertrophic cardiomyopathy, and restrictive cardiomyopathy. Although perhaps blurred by common co-morbidities, it is somewhat

surprising that with such fundamentally different etiologies, it is still unclear if iHF and niHF differ in terms of cardiomyopathy-related cellular and non-cellular metabolic adaptations (47, 48) and mitochondrial free radical production (9, 47). Indeed, although several studies have speculated that CAD-induced chronic ischemia may have quite a different effect on cardiac mitochondrial function than non-ischemic tissue in the same heart (7, 48), the actual impact of HF etiology on mitochondrial function remains unknown.

These unsolved issues regarding the impact of HF etiology are likely, at least in part, due to the limited opportunities to obtain human cardiac muscle, a failure to differentiate between HF etiologies, and may be further blurred by common co-morbidities. Therefore, using normal HdH as a reference and without the inclusion of patients with Diabetes, to minimize the effect of this co-morbidity, this study sought to examine the impact of HF etiology on mitochondrial function. Specifically, a comparison of mitochondrial function in cardiac muscle from patients with iHF and niHF was performed to test the following hypotheses: First, mitochondria in cardiac muscle from patients with HF would exhibit significantly more attenuated OXPHOS than the HdH. Second, although, in mass-specific terms, cardiac muscle from the iHF would exhibit lower OXPHOS than from patients with niHF, this difference would be negated by normalizing for mitochondrial content. However, finally, it was additionally hypothesized that accompanying this reduced mitochondrial content in iHF would be evidence of greater uncoupled respiration, leading to attenuated OXPHOS efficiency and greater free radical production in comparison to patients with niHF. Recognizing that large-scale clinical trials have reported that iHF and niHF respond differently to interventional drug

therapies(8, 15), the assessment of potential differences in mitochondrial function due to etiology is important in terms of accurately targeting specific patients with the appropriate treatment including pharmacological options[14, 15]

Methods

Subjects

Cardiac tissue was collected from 35 patients with HF and 4 healthy donors. HF patients were classified as iHF (n=17, evidence of CAD) and niHF (n=18, no evidence of CAD, evidence of dilated cardiomyopathy, hypertrophic cardiomyopathy, or restrictive cardiomyopathy). Cardiac tissue from patients with iHF and niHF was harvested from the apex of left ventricle during either heart transplantation or left ventricular assist device (LVAD) implantation surgeries. All patients with HF were free from diabetes and there was no evidence of insulin resistance. The tissue from the HdH, not allocated for heart transplantation due to non-cardiac issues (e.g. heart size, incarceration, etc.) (36) was also harvested from the apex of left ventricle. Subjects or their legal representative (HdH) provided informed consent, and the study protocols were approved by the University of Utah and the Veteran's Affairs Medical Center Institutional Review Boards. All protocols were carried out in accordance with the Declaration of Helsinki.

Muscle fiber permeabilizing and mitochondrial respiration assessments

Following cardiac tissue collection, fat and connective tissue were removed from the cardiac muscle in pre cooled buffer A (in mM: 2.77 CaK_2EGTA, 7.23 K_2EGTA, 6.56 $MgCl_2$, 0.5 dithiothreitol (DTT), 50 K-MES, 20 imidazol, 20 taurine, 5.77 Na_2ATP, 15

phosphocreatine, pH 7.1 at 4°C) and the sample remained in this solution until permeabilization (36).

All muscle samples were stored in precooled buffer A for no more than 30 min prior to the commencement of the permeabilizing procedures (25). Permeablizing procedures have been previously reported by our group (36). Briefly, muscle fibers were teased apart to increase permeability of the membrane and avoid limited diffusion of the substrates. After mild shaking for 30 min in buffer A with saponin (50 g/ml), the muscle was rinsed twice in buffer B (in mM: 2.77 CaK_2EGTA, 7.23 K_2EGTA, 6.56 $MgCl_2$, 0.5 DTT, 20 imidazole, 5.77 ATP, 15 PCr, 50 K-MES, 20 taurine, pH 7.0) for 10 min(36).

Mitochondrial respiratory oxygen (O_2) flux (JO_2) was assessed by a Clark type, high resolution, respirometer (Oxygraph, Hansatech, Kings Lynn, UK). The permeabilized muscle fibers (2-4 mg wet wt) were incubated in the respirometer with 2 ml of buffer B while being continuously stirred at 37°C. First, baseline muscle respiration was recorded, in the absence of respiratory substrates. To assess the function of each mitochondrial complex, O_2 consumption was assessed using a series of respiratory substrates and inhibitors in the following order and final concentrations in the chamber: glutamate-malate (2:10 mM), ADP (5 mM), succinate (10 mM), cytochrome c (10 μM), rotenone (0.5 μM), antimycin-A (2.5 μM), oligomycin (2 μg/ml), N,N,N,N-tetramethyl-p-phenylenediamine (TMPD)-ascorbate (2:0.5 mM) (36). This allowed the determination of 1) Complex I state 2 respiration, the non-phosphorylating resting state that provides an index of proton leak, assessed in the presence of malate + glutamate, 2) Complex I, state 3 respiration, the ADP-activated state of oxidative phosphorylation, assessed in the presence of glutamate + malate + ADP, 3) Complex I+II, state 3 respiration, assessed in

the presence of glutamate + malate + ADP + succinate 4) Complex II, state 3 respiration, assessed in the presence of glutamate + malate + ADP + succinate + rotenone 5) Complex IV respiration, assessed by the blocking of Complex 3 (antimycin A) and Complex V (oligomycin) followed by the addition of TMPD + ascorbate (36).

In each condition, respiration rate was recorded for 3-4 min and the average of the last 30 seconds was used for data analysis. Mitochondrial membrane integrity was evaluated by cytochrome c induction. The rate of O_2 consumption was measured in pmol of O_2 per second and then expressed relative to muscle sample mass (pmol·sec·wet weight). These respiration rates were further normalized by CS activity. The respiratory control ratio (RCR) was calculated as Complex I+II state 3/state 2 respiration. The substrate control ratio (SCR), an index of substrate utilization capacity at Complex II, was calculated as Complex I+II state 3/complex I state 3 (36).

Biochemical and histochemical analyses

Citrate synthase activity (CSA), an indicator of mitochondrial content (26), was measured by as previously described (26, 38). Briefly, the frozen cardiac muscle, that had been used for mitochondrial respiration measurements, was homogenized in extraction buffer (50 mM triethanolamine and 1 mM EDTA) using a bead homogenizer (BioSystems, Hamburg, Germany). CSA was assessed by spectrophotometry at 412 nm of the absorbance of light while incubated with 200 µl of reaction buffer (2 µm acetyl-Coa, 200 µm 5,5'-dithiobis- (2-nitrobenzoic acid) (DTNB), 350 µM oxaloacetic acid, and 0.1% Triton-X) (36).

Mitochondrial DNA (mtDNA)

The frozen cardiac muscle was homogenized and the DNA dissolved in 100 μl of Tris-EDTA. Five µl of a 50 times DNA dilution was used for PCR amplification with QuantiTect SYBR Green PCR Master Mix (Qiagen, Hilden, Germany) containing 0.5 µM of each primer in a total volume of 25 μl. Levels of mtDNA and genomic DNA (gDNA), as determined by *albumin* content, was assessed by real-time PCR using a Biorad IQ real-time PCR machine (Stratagene, La Jolla, CA, USA), as previously described (41). The copy number of mtDNA and gDNA was used as an estimate of mitochondrial concentration in cardiac muscle (24).

Free radical measurements in cardiac muscle from iHF and niHF

Total free radical levels in the cardiac muscle from both the iHF and niHF patients was assessed by 2'-7'-dichlorofluorescein-diacetate (DCFDA) fluorescence. Tissue was homogenized in 0.05% Trypsin-EDTA (Invitrogen, Carlsbad, CA) using a motor-driven tissue homogenizer (Qiagen Inc., Valencia, CA) and incubated for 30 min at 37 ºC. Following centrifugation (5 min at 14,000 g), muscle lysates were incubated in 5 mM DCF (invitrogen, Carlsbad, CA) dissolved in DMEM (Invitrogen, Carlsbad, CA) for 30 min at 37 ºC. Samples were centrifuged at 14,000 g for 5 min, pellets were re-suspended in 200 µl lysis buffer (50 mmol/L Hepes, 150 mmol/L NaCl, 10% Glycerol, 1% Triton X-100, 1.5 mmol/L $MgCl_2$, 1 mmol/L, EGTA, 10 mmol/L Sodium Pyrophosphate, 100 mmol/L Sodium Fluoride and 100 µmol/L Sodium Vanadate, 1 mmol/L PMSF, 10 µg/ml Aprotinin, and 10 µg/ml Leupeptin) and incubated for 10 min at 4º C under constant agitation. Samples were then centrifuged (14,000 g for 5 min) and

the supernatants transferred to a 96-well plate and fluorescence intensity was measured using a fluorescence plate reader (Biotek Instrument Inc, Winooski, USA). Data were normalized to protein content and the data were expressed as fold change (22).

To directly assess mitochondrial derived superoxide production, EPR spectroscopy (EMX X-band Bruker, Manning Park Billerica, MA) was performed on frozen cardiac tissue samples from the patients with iHF and niHF, utilizing mitoTempo–H probes (0.5 mM, Enzo Life Science, Inc.), as previously described (12, 18).

Statistical analyses

One way ANOVA was performed using SPSS version 18 (SPSS Inc., Chicago, IL, USA) to detect differences in respiration, mitochondrial content, and reactive O_2 species production. If a significance difference was detected, a Tukey's post hoc test was used to identify this difference. Correlations between CSA, Complex IV respiration, and mtDNA coppy number were assessed with a Pearson product moment correlation. For all analyses, a *P* value of <0.05 was considered significantly different. All data are expressed as mean ± SEM.

Results

Subject characteristics

The subject characteristics, disease conditions, and medications of the iHF and niHF patients are displayed in Table 3.1. The iHF and niHF patients were well-matched for age, physical, and blood chemistry characteristics. The 4 HdH came from 1 male and 3 females with an average age of 52±3 yrs. Thus, the HdH were of a similar age as the

patients with iHF and niHF; however, other physical and blood chemistry characteristics were not available for these individuals.

Oxidative phosphorylation capacity (OXPHOS) and mitochondrial content

As illustrated in Figure 3.1, Complex I, Complex II, and Complex I+II state 3 respiration rates (OXPHOS), expressed per unit of wet weight, was consistently attenuated in both the iHF and niHF compared to the HdH ($P<0.05$). However, OXPHOS was also consistently attenuated in the iHF compared to niHF ($P<0.05$) (Figure 3.1). Markers of mitochondrial content, CSA, Complex IV reparation, and mtDNA all fell consistently from HdH to niHF, to iHF (Figure 3.2) and these measures were significantly correlated with each other to varying extents (CSA vs mtDNA: $r = 0.36$, $P<0.05$; Complex IV respiration vs mtDNA: $r = 0.42$, $P<0.05$; CSA vs Complex 4 respiration: $r =0.8$, $P<0.05$, Figure 3.3A). When mitochondrial Complex I, Complex II, and Complex I+II state 3 respiration were normalized by CSA, OXPHOS for the HdH remained significantly greater than both iHF and niHF, but the difference between iHF and niHF was negated (Figure 3.3B). Also, SCR, an index of Complex II substrate utilization capacity, was not different across iHF, niHF, and HdH (1.3± 0.1, 1.3±0.1, 1.6±0.3, and $P= 0.1$, respectively).

Non-phosphorylating respiration and RCR

Complex I state 2 respiration, an index of *non-phosphorylating* proton leak, was significantly greater in iHF than niHF, but was still significantly greater in niHF than the HdH ($P<0.05$) (Figure 3.4A), and these differences were enhanced by normalizing for

CSA (Figure 3.4B). As both complex I state 2 respiration normalized by tissue wet weight and CSA fell from iHF to niHF to HdH ($P<0.05$), when the RCR (Complex I+II, state 3 respiration/Complex I, state 2 respiration) was calculated, there was a progressive and significant decline in RCR from HdH to niHF, to iHF ($P<0.05$) (Figure 3.4C).

Cardiac tissue free radical levels in iHF and niHF

The total free radical level, assessed by DCFDA fluorescence, was greater in iHF compared to niHF (Figure 3.5A). Similarly, the level of mitochondrial-specific superoxide, assessed by EPR spectroscopy, was significantly greater in iHF compared to niHF (Figure 5B).

Discussion

The study sought to examine the impact of HF etiology on mitochondrial respiratory function and, to our knowledge, this is the first investigation to comprehensively assess these processes in both iHF and niHF, compared to HdH. Consequently, there are several novel findings of this study. First, expressed per mg of tissue, OXPHOS, assessed as Complex I, II, and I+II state 3 respiration and mitochondrial content, assessed by CSA, fell progressively from HdH to niHF, to iHF. Second, although still lower than HdH, normalization of OXPHOS by CSA negated the difference in mass-specific OXPHOS between iHF and niHF. Third, Complex I state 2 respiration increased progressively from HdH to niHF, to iHF, such that RCR fell progressively from HdH to niHF, to iHF. Finally, measurements of both total free radical concentration and mitochondrial derived superoxide levels were significantly greater in

iHF compared to niHF. Thus, the HF-related attenuation in OXPHOS actually appears to be independent of etiology when the lower mitochondrial content of iHF is taken into account. However, this study also reveals deleterious intrinsic mitochondrial changes in iHF, compared to niHF, including greater proton leak, attenuated OXPHOS efficiency, and greater free radical levels. Identifying these etiology-specific mitochondrial function differences is important so as to accurately target specific patients with the appropriate treatments.

The impact of etiology on OXPHOS

In the human heart, 90% of ATP is produced by **OXPHOS;** therefore, an attenuation of this process in HF constitutes a major concern in terms of cardiac energy production (13, 29, 33). Indeed, the current study confirms and extends previous work that has typically observed attenuated Complex I state 3 respiration in both animal models of HF and patients with HF (42, 44, 45, 47). Specifically, this study documents a HF-related attenuation in OXPHOS, per mg of tissue, assessed as Complex I, Complex II, and Complex I+II state 3 respiration, falling progressively from HdH to niHF, to iHF. Therefore, although previous studies have suggested that mitochondrial structure and function is not affected by HF etiology (20, 21, 44), the current findings clearly demonstrate that tissue mass specific OXPHOS is attenuated with HF in general, but that this limitation occurs to a greater extent in iHF compared to niHF. These conflicting results may be a consequence of the inclusion of co-morbidities such as diabetes and insulin resistance in prior studies, in contrast the current study excluded these confounding factors. Interestingly, when mass-specific OXPHOS was normalized by

CSA, a marker of mitochondrial content, considered valid in HF (19, 26, 27, 36), the differences in respiration between iHF and niHF were negated. This suggests that OXPHOS per unit of mitochondrial content is similar between iHF and niHF and the observed differences in respiration were due to differences in the quantity rather than the quality of mitochondria between etiologies, with iHF exhibiting lower mitochondrial content and therefore lower mass-specific OXPHOS.

Utilizing the innovative approach of examining mitochondrial respiration in cardiac tissue from ischemic and non-ischemic areas of the same heart, Stride et al. (47), inferred that iHF likely resulted in a mitochondrial defect that manifests predominantly at Complex II. However, the current findings suggest that the attenuated OXPHOS with HF, including complex II respiration, is attenuated as a consequence of lower mitochondrial content, and, therefore, this apparently limited function was no longer evident when respiration was normalized for CSA (Figure 3.3B). Additionally, in the current study, SCR, an index of substrate utilization capacity at Complex II, was similar between iHF and niHF, further supporting the conclusion that Complex II function is not specifically impacted by HF etiology.

Mitochondrial content in iHF and niHF

Interestingly, there is little accord as to the most appropriate and valid method to assess tissue mitochondrial content; however, suggested approaches include electron microscopy, CSA, mtDNA, and Complex IV respiration. Recently, by revealing a strong relationship between CSA, a reasonably well-accepted method, and Complex IV respiration, in healthy cardiac, skeletal, and vascular smooth muscle, our group

concluded that Complex IV respiration is an acceptable marker of mitochondrial content (26, 36). The current data extend this previous conclusion to diseased tissue because across HdH, niHF, and iHG, complex IV was again highly correlated with CSA (Figure 3.3A). Additionally, mtDNA copy number revealed a similar pattern of reduction from HdH to niHF, to iHG as did CSA and Complex IV respiration (Figure 3.2). However, upon closer inspection, it was apparent that by far the best agreement amongst these measures of mitochondrial content was between CSA and Complex IV respiration (Figure 3.3A), adding a degree of construct validity to both indices. In terms of HF, these findings of diminished mitochondrial content are well aligned with a previous study by Lemiex et al., (27) which reported a decrement in Complex IV enzyme activity in cardiac tissue from patients with HF compared to healthy controls. Therefore, in combination with prior work, the current findings suggest that both CSA and Complex IV respiration are viable markers of mitochondrial content in both healthy and diseased cardiac muscle.

Perhaps, mechanistically, as a result of diminished mitochondrial calcium handling capacity, the activation of caspase-3, and the triggering of apoptosis (37, 53), several studies have reported a reduction in mitochondrial content in the cardiac tissue of patients with end-stage HF (Class IV). However, the specific impact of HF etiology on mitochondrial content has not been investigated (17, 21, 30, 40). As already noted, recently, Stride et al. (47) investigated the impact of ischemia on mitochondrial content by examining ischemic and non-ischemic regions within the same heart. The authors determined that, in comparison to well-perfused regions of the heart, mitochondrial content was reduced in the chronically ischemic areas, implicating a link between chronic ischemia and reduced mitochondrial content (47). The current study supports this

contention by clearly revealing distinct etiology-dependent differences in mitochondrial content between iHF and niHF, with iHF resulting in reduced mitochondrial density (Figure. 3.2). The specific mechanism underlying the reduced mitochondrial content in iHF compared to niHF remains unclear; however, this finding is likely related to the impaired tissue oxygenation associated with chronic ischemia. This could result in chronic low–grade inflammation, the up-regulation of the pro-inflammatory cytokine tumor necrosis factor-α, and attenuated mitochondrial biogenesis, as evidenced by reduced PGC-1α (16, 34). Additionally, impaired mitochondrial oxygenation attenuates respiratory complex function and adenine nucleotide translocase which may lead to membrane dysfunction and, eventually, cell death (52). Another, outcome of chronic ischemia is the accumulation of collagen and the development of fibrosis, reducing mitochondrial density per tissue mass, a phenomenon which has been documented to be more prevalent in iHF than niHF (28, 39). Each of these factors deserve further attention to determine the mechanism responsible for the observed reduction in mitochondrial content in iHF compared to niHF

Non-phosphorylating respiration and RCR in iHF and niHF

The proton motive force, the proton gradient between the matrix and the intermembrane space of the mitochondria, facilitates ATP production (49) and in combination with non-phosphorylating proton conductance regulates the kinetics and efficiency of mitochondrial respiration (2, 36). Interestingly, in the current study, despite greater mitochondrial content in niHF compared to iHF, tissue mass-specific Complex I state 2 respiration, an indicator of proton leak, was greater in iHF compared to niHF

(Figure 3.4A). Therefore, when normalized for CSA, this elevated Complex I state 2 respiration in the iHF compared niHF was not only maintained, but was exaggerated, confirming that proton leak per mitochondrial content was greater in iHF compared to niHF. This general finding, of greater proton leak in HF, is well-aligned with previous studies suggesting greater mitochondrial uncoupling with mitochondrial membrane damage and subsequent reductions in ATP production in mice with HF (1, 2, 52). Furthermore, the current etiology specific findings suggest that chronic ischemia increases mitochondrial H^+ ion membrane permeability that likely contributes to mitochondrial dysfunction. This difference in proton leak may be a fundamental difference which characterizes HF etiologies (4, 5) (Figure 3.4A and 3.4B). Additionally, the RCR, the ratio of Complex I+II state 3 to Complex I state 2 respiration, provides an index of mitochondrial dysfunction, with a low RCR reflecting defects that lead to OXPHOS inefficiency (3, 36). The current findings reveal a significantly lower RCR in iHF compared to niHF. This attenuated RCR in iHF compared to niHF provides additional novel evidence that HF etiology results in physiologically significant alterations in mitochondrial proton leak. Therefore, this study reveals that iHF exhibits an even greater attenuation in OXPHOS efficiency than niHF and this is predominantly due to distinct etiology-dependent uncoupled respiration.

Etiology specific free radical production in iHF and niHF

Previous studies have reported elevated free radicals in HF (6, 48); however, the source of these free radicals, mitochondrial or non-mitochondrial, is not clear. Additionally, these previous investigations are somewhat confounded by the use of acute

ischemia, in animal models, to induce free radical production (6, 48). The current study utilized DCFDA to assess basal free radical levels in cardiac muscle from patients with iHF and niHF and revealed a greater total free radical level in the tissue from the patients with iHF (Figure 3.5A). Furthermore, the direct assessment of mitochondrial derived superoxide levels, utilizing the mitoTempo-H spin probe and EPR spectroscopy, revealed greater free radical levels in iHF compared to niHF (Figure 3.5B). Although speculative, it is tempting to imply that this may be a consequence of both a reduction in mitochondrial content and increased H^+ membrane permeability in iHF compared to niHF (34, 51).

Previously, Stride et al. (47) measured greater free radical production in ischemic compared to non-ischemic regions of the same heart in patients with HF. In agreement with this study, the current findings suggest that chronic ischemia may be an important factor contributing to increased mitochondrial-derived free radical production in iHF. Potential mechanisms responsible for this augmented free radical production include attenuated Complex III function and diminished coenzyme Q binding protein (COQ10), both of which would result in a "bottleneck" for electron transport through the respiratory chain, allowing more time for free radical production (47, 48). However, it is still somewhat controversial as to whether increased proton leak decreases free radical production due to decreased proton motive force and the subsequent attenuation of the coupled oxidative phosphorylation or if an increased proton leak reduces ATP/O_2 ratio (P/O ratio), leading to an increase free radical production due to a decreased integrity of mitochondrial membranes (46, 50). In actuality, the relationship between proton motive force and free radical formation is likely described by a U-shaped curve and thus it will

not be possible to discern a single, simple, answer to this complex issue (10). Nonetheless, this study has identified a greater total and mitochondrial-derived free radical production in the cardiac muscle of patients with iHF compared to niHF, revealing a clear etiology-dependent difference.

Summary

The HF-related attenuation in OXPHOS appears to be independent of etiology when the lower mitochondrial content of iHF, compared to niHF, is taken into account. However, there are also deleterious intrinsic mitochondrial changes in iHF, compared to niHF, including greater proton leak, attenuated OXPHOS efficiency, and greater free radical levels. Identifying these etiology-specific mitochondrial function differences is important so as to accurately target specific patients with the appropriate treatments.

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Table 3.1. Characteristic of the ischemic and non-ischemic heart failure (HF) patients

	Ischemic HF (n=17)	Non- ischemic HF (n=18)
Age (years)	57 ± 3	53 ± 4
Gender (M/F)	14/3	14/4
BMI ($kg/m^{2)}$	27.1	27.2
Systolic blood pressure (mm Hg)	115±3	112±4
Diastolic blood pressure (mm Hg)	74±2	72±3
NYHA classification: I-IV (n)	III(1)-IV(16)	III (2)-IV(16)
LVEF (%)	~21%	~24%
Type 2 diabetes as a co-morbidity	None	None
Medications		
ACE inhibitor	14/17	15/18
angiotensin antagonist	15/17	15/18
Beta- blocker	7/17	11/18
Diuretics	16/17	17/18
Statins	10/17	9/18
Digoxin	11/17	11/18
Inotropic support	7/17	3/18
Plasma biochemistry		
Total Cholesterol, mmol/l	160±10	150±16
HDL (mg/dL)	40±1	42±2
LDL (mg/dL)	110±2	99±2
Triglyceride (mg/dL)	99±2	108±2
Glucose (mg/dL)	100±4	99±3
Hematocrit (%)	40±2	39±2
RBC (M/uL)	4.3±0.2	4.2±0.7
WBC (K uL)	8.9±0.2	7.7±0.4

BMI: body mass index; NYHA: New York Heart Association; LVEF: left ventricular ejection fraction; Type II DM: type II diabetes mellitus; ACE inhibitor: angiotensin converting enzyme inhibitor; HDL: high-density lipoprotein; LDL: low-density lipoprotein; RBC: red blood cells; WBC: white blood cells. Data presented as mean ± SE.

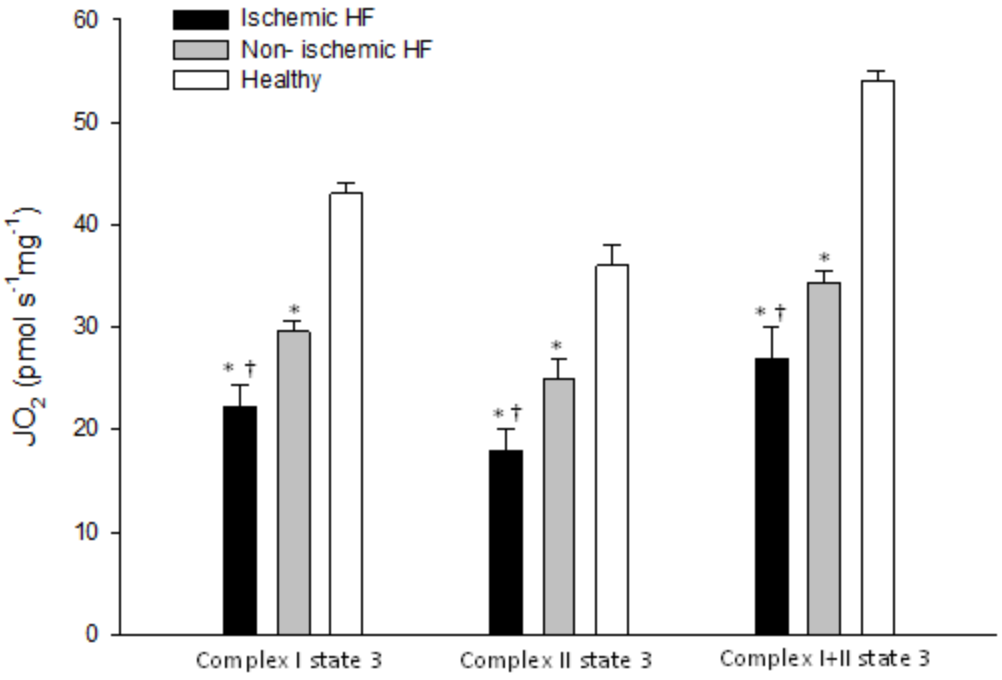

Figure 3.1. Oxidative phosphorylation capacity assessed as Complex I, Complex II, and Complex I+II state 3 respiration in the cardiac muscle of healthy hearts and patients with ishchemic and non-ischemic heart failure (HF). JO_2, O_2 flux. * Significantly different from healthy hearts, $P<0.05$; † Significantly different from non-ischemic HF, $P<0.05$. Data presented as mean ± SE.

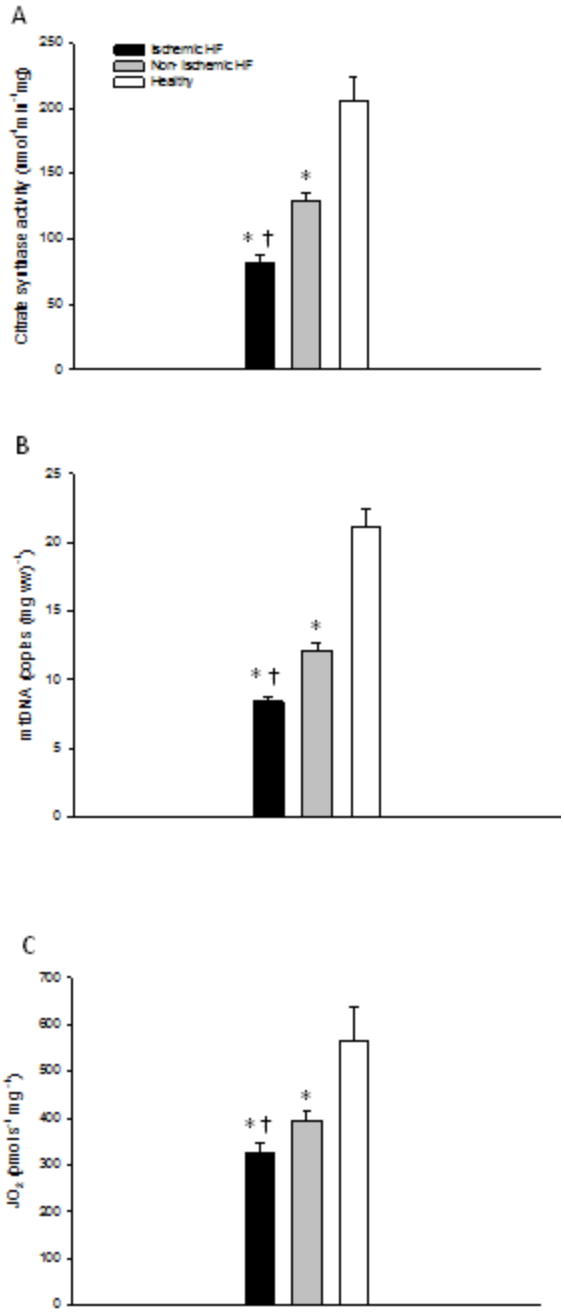

Figure 3.2. Markers of mitochondrial content in cardiac muscle from healthy hearts, and patients with ischemic and non-ischemic heart failure (HF). A: Citrate synthase activity, B: mitochondrial DNA copy number, and C: Complex IV respiration. JO_2, O_2 flux. * Significantly different from healthy hearts, $P<0.05$; † Significantly different from non-ischemic HF, $P<0.05$; mtDNA, mitochondrial deoxyribonucleic acid. Data presented as mean ± SE.

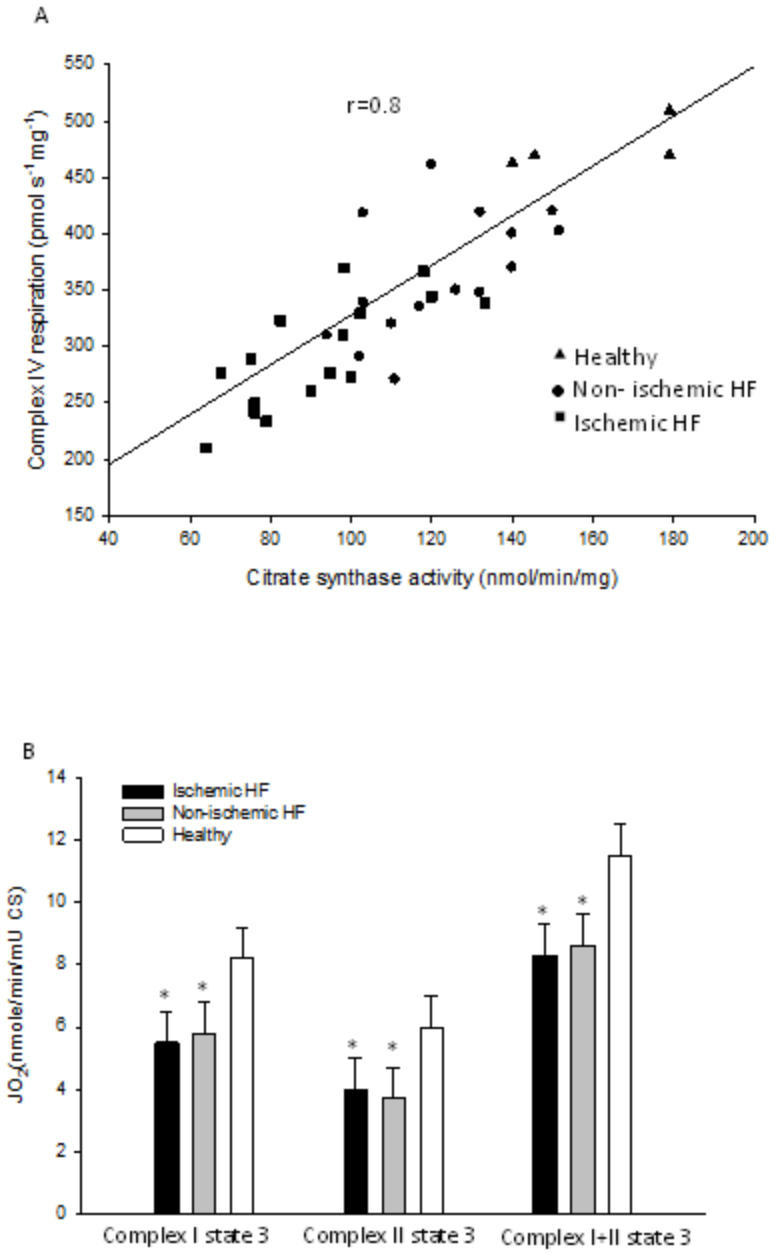

Figure 3.3. The significant relationship between citrate synthase activity (CSA) and Complex IV respiration (A) and oxidative phosphorylation capacity normalized by CSA (B) in cardiac muscle from healthy hearts and patients with ischemic and non-ischemic heart failure (HF). * Significantly different from the healthy hearts**,** P<0.05. Healthy hearts, triangles; non-ischemic HF, circles; ischemic HF, squares. Data presented as mean ± SE in panel

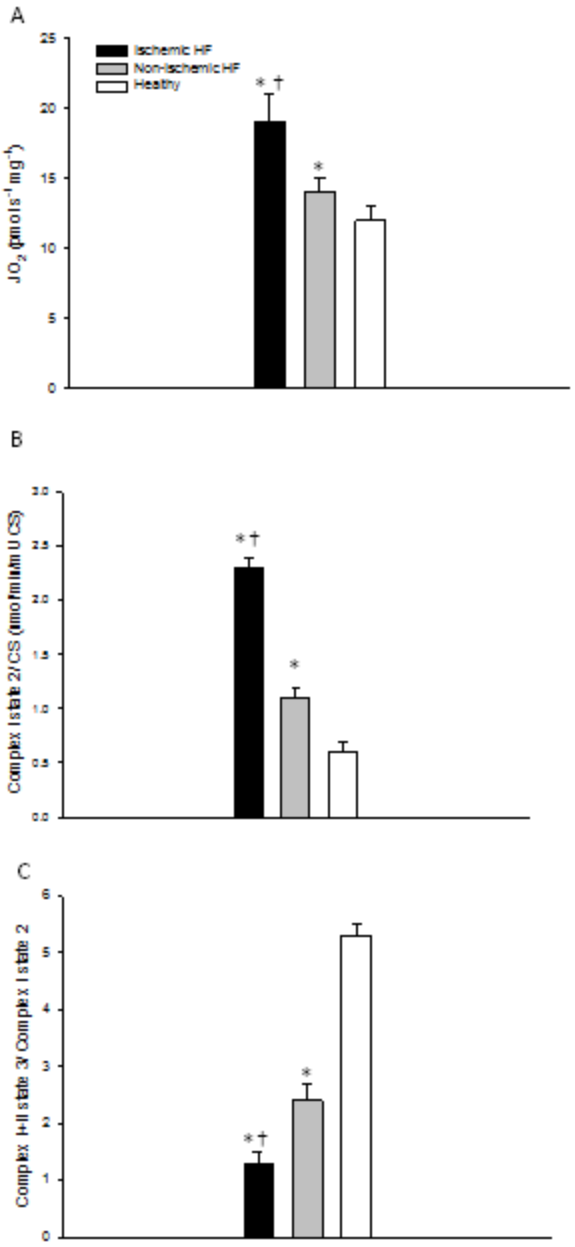

Figure 3.4. Uncoupled mitochondrial respiration (complex I state 2) normalized by tissue wet weight (A) and then by citrate synthase activity (B), and respiratory control ratio (RCR, complex I+II state 3 / complex I state 2 respiration) (C) in cardiac muscle from healthy hearts and patients with ischemic and non-ischemic heart failure (HF). * Significantly different from healthy hearts, $P<0.05$; † Significantly different from non-ischemic HF, $P<0.05$. Data presented as mean ± SE.

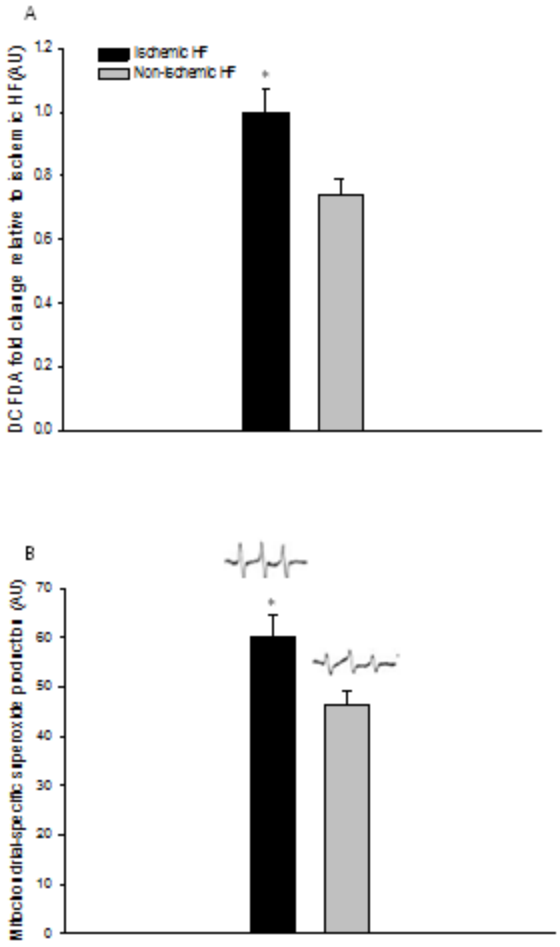

Figure 3.5. Basal free radical levels in cardiac muscle from patients with ischemic heart failure (HF) and non-ischemic HF. A: Total free radicals measured with, 2'-7' dichlorofluorecein diacetae (DCFDA) fluorescence in whole-tissue extract. Data presented as fold change relative to Ischemic HF. B: Mitochondrial-derived superoxide levels measured in frozen cardiac muscle utilizing the mitoTempo-H spin probe and electron paramagnetic resonance spectroscopy. * Significantly different from non-ischemic HF, $P < 0.05$. Data presented as mean ± SE.

CHAPTER 4

CARDIAC, SKELETAL, AND SMOOTH MUSCLE MITOCHONDRIAL RESPIRATION: ARE ALL MITOCHONDRIA CREASED EQUAL?

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Cardiac, skeletal, and smooth muscle mitochondrial respiration: are all mitochondria created equal?

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Park SY, Gifford JR, Andtbacka RH, Trinity JD, Hyngstrom JR, Garten RS, Diakos NA, Ives SJ, Dela F, Larsen S, Drakos S, Richardson RS. Cardiac, skeletal, and smooth muscle mitochondrial respiration: are all mitochondria created equal? *Am J Physiol Heart Circ Physiol* 307: H346–H352, 2014. First published June 6, 2014; doi:10.1152/ajpheart.00227.2014.—Unlike cardiac and skeletal muscle, little is known about vascular smooth muscle mitochondrial respiration. Therefore, the present study examined mitochondrial respiratory rates in smooth muscle of healthy human feed arteries and compared with that of healthy cardiac and skeletal muscles. Cardiac, skeletal, and smooth muscles were harvested from a total of 22 subjects (53 ± 6 yr), and mitochondrial respiration was assessed in permeabilized fibers. Complex I + II, state 3 respiration, an index of oxidative phosphorylation capacity, fell progressively from cardiac to skeletal to smooth muscles (54 ± 1, 39 ± 4, and 15 ± 1 pmol·s^{-1}·mg^{-1}, $P < 0.05$, respectively). Citrate synthase (CS) activity, an index of mitochondrial density, also fell progressively from cardiac to skeletal to smooth muscles (222 ± 13, 115 ± 2, and 48 ± 2 μmol·g^{-1}·min^{-1}, $P < 0.05$, respectively). Thus, when respiration rates were normalized by CS (respiration per mitochondrial content), oxidative phosphorylation capacity was no longer different between the three muscle types. Interestingly, complex I state 2 normalized for CS activity, an index of nonphosphorylating respiration per mitochondrial content, increased progressively from cardiac to skeletal to smooth muscles, such that the respiratory control ratio, state 3/state 2 respiration, fell progressively from cardiac to skeletal to smooth muscles (5.3 ± 0.7, 3.2 ± 0.4, and 1.6 ± 0.3 pmol·s^{-1}·mg^{-1}, $P < 0.05$, respectively). Thus, although oxidative phosphorylation capacity per mitochondrial content in cardiac, skeletal, and smooth muscles suggest all mitochondria are created equal, the contrasting respiratory control ratio and nonphosphorylating respiration highlight the existence of intrinsic functional differences between these muscle mitochondria. This likely influences the efficiency of oxidative phosphorylation and could potentially alter ROS production.

oxidative phosphorylation capacity; respiratory control ratio; feed arteries

THE INTEGRATED FUNCTION of cardiac, skeletal, and vascular smooth muscles is essential for O_2 delivery and utilization, especially during exercise, when synchronicity can determine capacity. Cardiac muscle produces the driving force to convectively transport blood-borne O_2 to the periphery, where skeletal muscle uses this O_2 for the metabolic requirements of locomotion (35, 36). Smooth muscle, the major component of the arterial system, dictates the distribution of blood flow and O_2 transport, dependent on need (6, 33). Each of these distinct muscle tissues contain mitochondria, which consume O_2 and produce ATP through cellular respiration. Interestingly, although mitochondrial respiration of both cardiac and skeletal muscles has been studied extensively in health and disease (4, 5, 31, 32, 39), little is known about smooth muscle mitochondrial respiration (42).

The heart is a vital organ with a high metabolic demand and is subsequently rich in mitochondria, with these mitochondria accounting for ~35% of the volume of cardiac tissue (39) and generating up to 90% of ATP requirements by β-oxidation at rest (21, 28, 30). Attenuated coupled respiration and an increase in uncoupled respiration in cardiac muscle are common indicators of heart disease (4). Indeed, diseased hearts often exhibit decreased oxidative phosphorylation, secondary to both reduced mitochondrial enzymes and content (27, 47), and excessive mitochondrial free radical production in human and animal models (16, 38). In skeletal muscle, due to the high metabolic requirements for locomotion, mitochondrial ATP production is also certainly important, with typically 3–8% of the skeletal muscle volume being mitochondria (23), but this is highly dependent on physical activity. Additionally, studies have revealed that a reduction in skeletal muscle mitochondrial oxidative phosphorylation capacity and/or volume may contribute to muscle dysfunction (11–13). Therefore, understanding and characterizing mitochondrial function in both cardiac and skeletal muscles has implications in both health and disease.

Although far less well studied, the mitochondria within smooth muscle are thought to play a role in maintaining vascular tone, facilitating cellular transport, and producing energy for vascular cell secretion (42, 46). These mitochondria typically comprise 3~5% of the smooth muscle cell volume (3). Until recently, little was known about the role of vascular smooth muscle mitochondria in terms of vascular function and disease. Now, new evidence suggests the potential importance of mitochondrial function in the development of vascular diseases (14, 43, 45). However, it is important to note that most of these studies used mitochondrial protein expression or

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protein activity to estimate mitochondrial function rather than actual measurements of mitochondrial respiratory capacity, and, hence, direct assessments of mitochondrial respiration in vascular smooth muscle are still lacking. Indeed, there has yet to be a comprehensive assessment of mitochondrial respiratory rate in smooth muscle and a comparison of this function with cardiac and skeletal muscles.

Recognizing the lack of data specific to mitochondrial function in smooth muscle of the vasculature, the present study sought to assess mitochondrial respiration in smooth muscle of skeletal muscle feed arteries and compare this with that of cardiac and skeletal muscles. Due to the anticipated differences in mitochondrial density between cardiac, skeletal, and smooth muscles, in conjunction with the vastly different functional requirements of each, it was hypothesized that, on a mitochondrion-to-mitochondrion basis, respiratory function would be very similar. This would indicate that all muscle mitochondria are created equal in terms of respiration, regardless of origin.

METHODS

Subjects. A total of 22 subjects (17 men and 5 women) participated in this study. Cardiac muscle was harvested from four subjects (2 men and 2 women), skeletal muscle was harvested from nine subjects (9 men), and skeletal muscle feed arteries were harvested from nine subjects (6 men and 3 women). All subjects were free from overt cardiovascular disease, and there was no evidence of involvement in regular exercise, as determined by interviews and medical records. Although none of the subjects were taking medications recognized to alter mitochondrial function, cardiac and skeletal muscle feed artery donors were anesthetized using a standard protocol that included propofol, fentanyl, benzodiazepines, and vecuronium bromide dosed according to the patient's body weight, whereas lidocaine was used for local anesthesia of subjects donating skeletal muscle. Subjects or their legal representative (cardiac muscle donors) provided informed consent, and study protocols were approved by the University of Utah and Veteran's Affairs Medical Center Institutional Review Boards.

Cardiac muscle. Cardiac muscle was harvested from the left ventricular apex of normal donor hearts, not allocated for heart transplantation due to noncardiac issues (e.g., heart size, incarceration, etc.). Fat and connective tissue were removed from cardiac muscle in precooled *buffer A* [containing (in mM) 2.77 CaK_2EGTA, 7.23 K_2EGTA, 6.56 $MgCl_2$, 0.5 DTT, 50 K-MES, 20 imidazol, 20 taurine, 5.77 Na_2ATP, and 15 phosphocreatine, pH 7.1 at 4°C], and the sample remained in this solution until permeablization.

Skeletal muscle. After local anesthesia (5–10 ml of 1% Lidocaine HCL, Hospira, Lake Forest, IL), skeletal muscle was harvested by the needle biopsy technique from the lateral aspect of the quadriceps muscle. Skeletal muscle was stored and dissected in precooled *buffer A*, and the sample remained in this solution until permeablization.

Skeletal muscle feed arteries. Human skeletal muscle feed arteries (~7 mg wet wt) were harvested as previously reported (20). Briefly, feed arteries (200–500 μm) were identified and harvested during melanoma-related surgeries in either the axillary and inguinal regions. Specifically, skeletal muscle feed arteries, supplying the serratus anterior, latissimus dorsi, quadriceps femoris, and hip adductor muscles, were harvested for this study. Skeletal muscle feed arteries were stored and dissected in precooled *buffer A*, and the sample remained in this solution until permeablization.

Preparation of permeabilized tissues. All muscle samples were stored in precooled *buffer A* for <30 min before the commencement of the permeabilizing procedures (22). Specifically, cardiac and skeletal muscle tissues were teased apart by needle tip to increase permeability of the membrane and avoid limited diffusion of the substrates. After mild shaking for 30 min (cardiac and skeletal muscles) and 40 min (vessels) in *buffer A* with saponin (5 mg/ml), the muscle was rinsed twice in *buffer B* [containing (in mM) 2.77 CaK_2EGTA, 7.23 K_2EGTA, 6.56 $MgCl_2$, 0.5 DTT, 20 imidazole, 5.77 ATP, 15 phosphocreatine, 50 K-MES, and 20 taurine, pH 7.0] for 10 min. Note that the optimum duration of permeabilization for vessel mitochondria (longer than cardiac and skeletal muscles) was determined by a series of pilot studies before this investigation. Specifically, four different permeablization time periods (20, 30, 40, and 50 min) were assessed, with the highest respiration rate occurring with the 40-min period, without membrane damage (cytochrme *c*). The optimal substrate concentration for vessel mitochondrial respiration was determined by titrating the ADP concentration in 1-mM increments (final chamber concentration: 1–6 mM), which revealed that an ADP concentration of 5 mM resulted in the highest respiration rate and that 6 mM did not facilitate any further increase.

Mitochondrial respiration. Mitochondrial respiratory O_2 flux was assessed with a Clark-type high-resolution Oxygraph respirometer (Hansatech, Kings Lynn, UK). Permeabilized muscle fibers (2–4 mg wet wt) were incubated in the respirometer with 2 ml *buffer B* while being continuously stirred at 37°C. First, baseline muscle respiration was recorded, in the absence of respiratory substrates. To assess the function of each mitochondrial complex, O_2 consumption was assessed with the addition of a series of respiratory substrates and inhibitors in the following order and final concentrations in the chamber: glutamate-malate (2:10 mM), ADP (5 mM), succinate (10 mM), cytochrome *c* (10 μM), rotenone (0.5 μM), antimycin-A (2.5 μM), oligomycin (2 μg/ml), and *N*,*N*,*N*′,*N*′-tetramethyl- *p*-phenylenediamine (TMPD)-ascorbate (2:0.5 mM). This allowed the determination of *1*) complex I state 2 respiration, the nonphosphorylating resting state that provides an index of proton leak, assessed in the presence of malate + glutamate; *2*) complex I, state 3 respiration, the ADP-activated state of oxidative phosphorylation, assessed in the presence of glutamate + malate + ADP; *3*) complex I + II, state 3 respiration, assessed in the presence of glutamate + malate + ADP + succinate; and *4*) complex IV respiration, assessed by blocking complex 3 (antimycin A) and complex 5 (oligomycin) followed by TMPD + ascorbate. O_2 consumption derived from autoxidation of TMPD and ascorbate was measured with the following method: first, complex III respiration was inhibited by antimycin A, and then complex IV respiration was measured. Complex IV respiration was then blocked by cyanide at the very low O_2 tension in the chamber to measure chemical respiration. Chemical and instrument-related respiration (without tissue) was subtracted from total complex IV respiration to calculate complex IV respiration.

In each condition, the respiration rate was recorded for 3 min, and the average of the last minute was used for data analysis. Mitochondrial membrane integrity was evaluated by cytochrome *c* induction. The rate of O_2 consumption was measured as picomoles of O_2 per second and then expressed relative to muscle sample mass (in $pmol \cdot s^{-1} \cdot mg\ wet\ wt^{-1}$). These respiration rates were further normalized by either citrate synthase (CS) activity or complex IV respiration. The respiratory control rate (RCR) was calculated by state 3/state 2 respiration, normalized for CS activity. The substrate control ratio for succinate was calculated as complex I + II state 3/complex I state 3.

CS activity. After the respiration measurements, the same muscle samples (3.5–4.5 mg wet wt) were homogenized with homogenization buffer [containing (in mM) 250 sucrose, 40 KCl, 2 EGTA, and 20 Tris·HCl] (Qiagen, Hilden, Germany). The CS activity assay was performed as previously described (31) and read with a spectrophotometer (Biotek Instrument).

Statistical analysis. One-way ANOVA was performed using SPSS (version 18, SPSS, Chicago, IL). If significance was detected, a Tukey post hoc test was used to identify the significant difference. For all analyses, a P value of <0.05 was considered significantly different. All data are expressed as means ± SE.

Table 1. *Subject characteristics*

	Cardiac Muscle	Skeletal Muscle	Smooth Muscle
Total number of subjects	4	9	9
Age, yr	52 ± 3	54 ± 3	52 ± 1
Height, cm	161 ± 15	174 ± 3	177 ± 4
Body mass, kg	57 ± 10	77 ± 10#	104 ± 7*†
Systolic blood pressure, mmHg		120 ± 2	121 ± 3
Diastolic blood pressure, mmHg		78 ± 2	77 ± 2
Mean arterial pressure, mmHg		92 ± 4	92 ± 5
Medications			
Over-the-counter analgesics		0/9	2/9
Ibuprofen or excedrin		0/9	2/9
Cardiovascular	0/4	0/9	0/9
Diabetic	0/4	0/9	0/9

Data are expressed as means ± SE or number of subjects (out of the total number). Note that users of cardiovascular (statin, β-blocker, angiotensin-converting enzyme inhibitor, diuretic, Ca^{2+} channel blocker, etc.) and diabetic (insulin, metformin, etc.) medications were excluded from the study. *$P < 0.05$, smooth muscle vs. cardiac and skeletal muscles; †$P < 0.05$, smooth muscle vs. skeletal muscle; #$P < 0.05$, skeletal muscle vs. cardiac muscle.

RESULTS

Subject characteristics. The ages of the subjects who provided cardiac muscles (52 ± 3 yr), skeletal muscles (54 ± 3 yr), and smooth muscles (52 ± 1 yr) were well matched across the groups. However, cardiac muscle donors were significantly shorter in stature (height: 161 ± 15 cm) and lighter (weight: 57 ± 10 kg) than skeletal muscle donors (height: 174 ± 3 and weight: 77 ± 10 kg) and smooth muscle donors (height: 177 ± 4 cm and weight: 104 ± 7 kg), who were also different from each other in terms of body weight (Table 1).

Oxidative phosphorylation. As shown in Fig. 1, when state 3 respiration rates for both complex I as well as complex I and II were expressed per unit of wet weight, oxidative phosphorylation capacity fell progressively from cardiac to skeletal to smooth muscles (complex I: 43 ± 0.1 vs. 32 ± 3 vs. 13 ± 2 pmol·s^{-1}·mg wet wt^{-1}, $P < 0.05$, respectively, and complex I and II: 54 ± 1 vs. 39 ± 4 vs. 15 ± 1 pmol·s^{-1}·mg wet wt^{-1}, $P < 0.05$, respectively). CS activity also fell from cardiac to skeletal to smooth muscles (222 ± 13, 115 ± 2, and 48 ± 2 μmol·min^{-1}·mg^{-1}, $P < 0.05$, respectively; Fig. 2*A*) and was therefore well correlated with both complex I ($r = 0.8$) and complex I + II, state 3 (Fig. 2*A*) respiration rates. When state 3 respiration for complex I as well as complex I and II was normalized by CS activity, mitochondrial respiration in these three muscle types, expressed simply as wet weight (Fig. 1), were no longer apparent, with cardiac, skeletal, and smooth muscles all exhibiting similar rates of oxidative phosphorylation (Fig. 2*B*). Complex IV was highly correlated with CS activity (Fig. 3*A*), and, thus, when state 3 respiration, assessed at either complex I or complex II, was normalized for complex IV respiration, sometimes considered an index of respiratory efficiency, there were no differences in this ratio across cardiac, skeletal, and smooth muscles (Fig. 3*B*). Also, the substrate control ratio was not different across cardiac, skeletal, and smooth muscles (1.3 ± 0.1, 1.3 ± 0.1, and 1.2 ± 0.3, respectively).

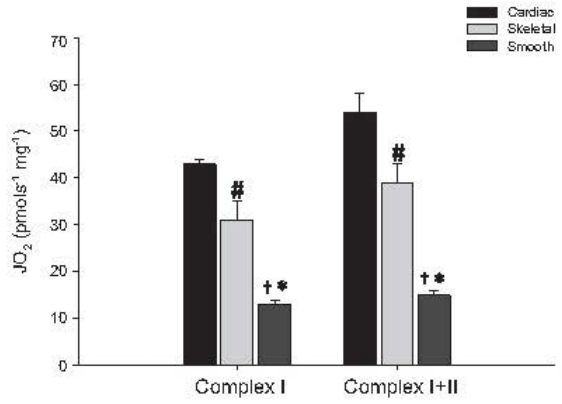
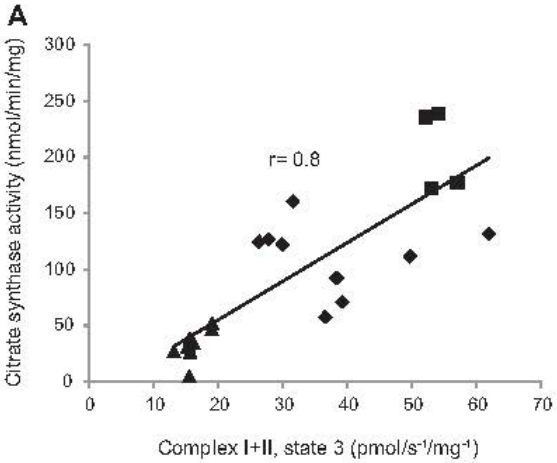


Fig. 1. Oxidative phosphorylation capacity in cardiac, skeletal, and vascular smooth muscles. Jo_2, O_2 flux. *$P < 0.05$, smooth muscle vs. cardiac and skeletal muscles; †$P < 0.05$, smooth muscle vs. skeletal muscle; #$P < 0.05$, skeletal muscle vs. cardiac muscle. Complex I, complex I state 3 respiration; complex I + II, complex I + II, state 3 respiration.



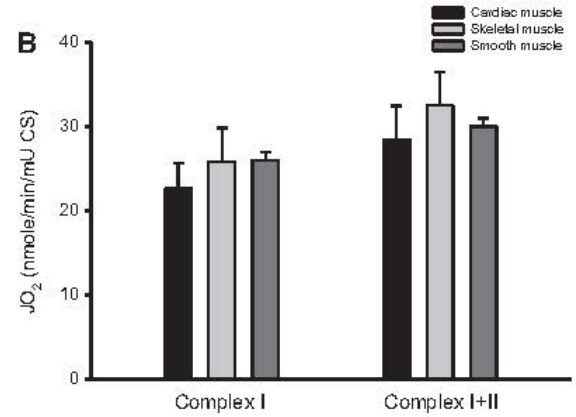


Fig. 2. *A*: relationship between citrate synthase (CS) activity and oxidative phosphorylation capacity (complex I + II, state 3) in cardiac muscle (squares), skeletal muscle (diamonds), and vascular smooth muscle (triangles). $P < 0.001$. *B*: oxidative phosphorylation capacity normalized by CS activity, a marker of mitochondrial density, in cardiac, skeletal, and vessel smooth muscles.

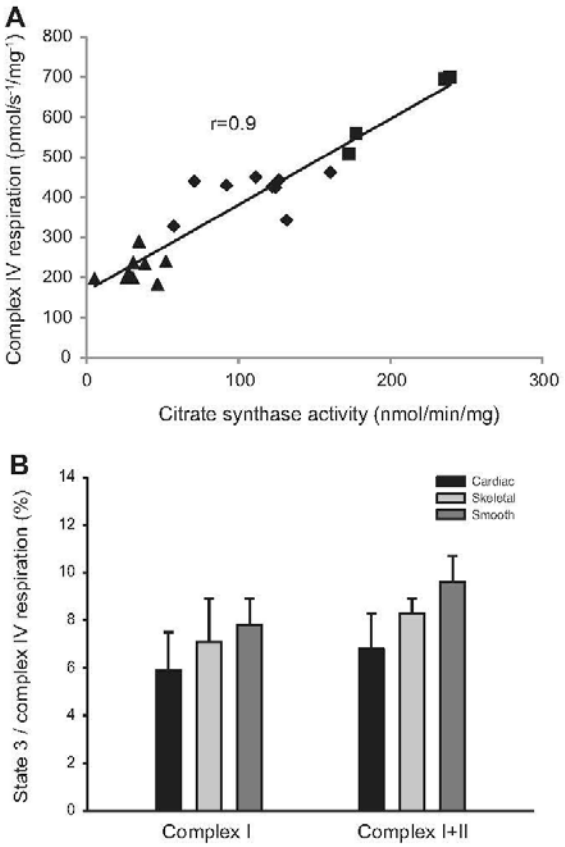


Fig. 3. *A*: relationship between CS activity and complex IV respiration (*N,N,N,N*-tetramethyl- *p*-phenylenediamine + ascorbate) in cardiac muscle (squares), skeletal muscle (diamonds), and vascular smooth muscle (triangles). $r = 0.9$, $P < 0.001$. *B*: oxidative phosphorylation capacity normalized by electron transport capacity.

Nonphosphorylating respiration and RCR. Complex I state 2 respiration, an index of proton leak, when expressed as wet weight, was not significantly different in cardiac, skeletal, and smooth muscles (Fig. 4*A*). When mitochondrial density was accounted for by normalizing these wet weight data by CS activity, there was a progressive rise in nonphosphorylating respiration or greater proton leak from cardiac to skeletal to smooth muscles (5.5 ± 0.5, 10.4 ± 0.8, and 16.4 ± 1.6 pmol·mg^{-1}·CS activity^{-1}, respectively; Fig. 4*B*). Thus, when the RCR (complex I + II, state 3 respiration/complex I, state 2 respiration) was calculated, there was a progressive fall from cardiac to skeletal to smooth muscles (5.3 ± 0.7, 3.2 ± 0.4, and 1.6 ± 0.3, respectively; Fig. 4*C*).

DISCUSSION

There are several novel findings of this study. First, vascular smooth muscle mitochondrial respiration can be successfully assessed using the same permeablized fiber approach routinely used with both cardiac and skeletal muscles. Second, likely a consequence of very different functional roles, oxidative respiratory capacity, measured as complex I + II, state 3 respiration and expressed as muscle weight, fell progressively from cardiac to skeletal to to smooth muscles. However, when respiration rates were normalized by CS activity, an index of mitochondrial content (23), allowing cardiac, skeletal, and smooth muscles to be compared in terms of mitochondrial content, complex I + II, state 3 respiration was very similar. Third, complex IV respiration across all muscles was very well correlated with CS activity, supporting previous suggestions that this is a good marker of mitochondrial content (23). Thus, normalization for complex IV respiration also annulled the differences in oxidative phosphorylation capacity between the three types of muscle. Finally, there were significant muscle-specific differences in both nonphosphorylating respiration and

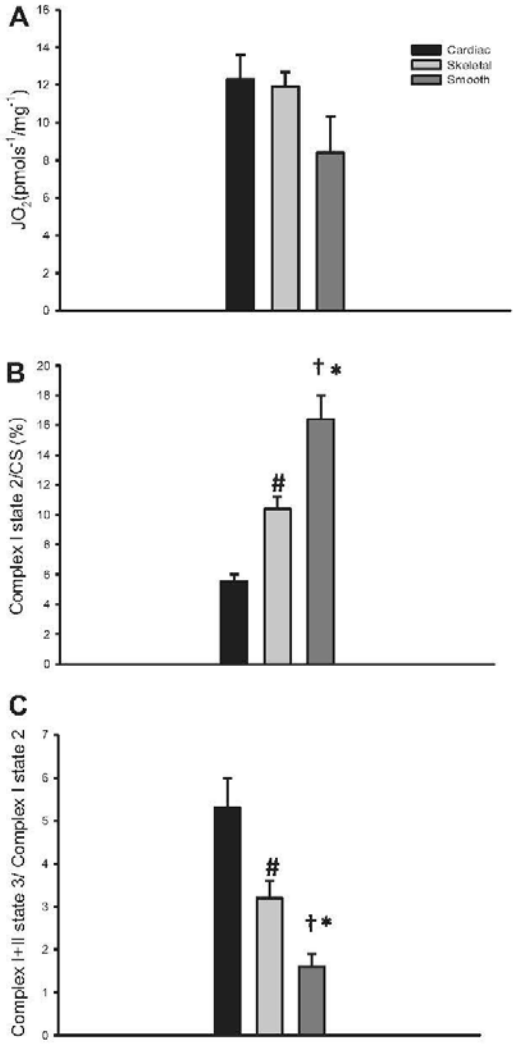


Fig. 4. *A*: complex I state 2 respiration. *B*: complex I state 2 respiration normalized by CS activity, a marker of mitochondrial density. *C*: respiratory control ratio, complex I + II, state 3 normalized by complex I state 2 in cardiac, skeletal, and smooth muscles. *$P < 0.05$, smooth muscle vs. cardiac and skeletal muscles; †$P < 0.05$, smooth muscle vs. skeletal muscle; #$P < 0.05$, skeletal muscle vs. cardiac muscle.

RCR. Therefore, although this study provides evidence that cardiac, skeletal, and smooth muscle mitochondria appear to be very similar in terms of oxidative phosphoylation capacity, RCR and nonphosphorylating respiration are certainly not similar. This highlights the existence of intrinsic functional differences between these muscle mitochondria, which likely influences the efficiency of oxidative phosphorylation and could potentially alter ROS production.

Cardiac, skeletal, and smooth muscle structure and function. Although both cardiac and skeletal muscles are striated, skeletal muscle is composed of slow (type I) and fast (type II) fibers, whereas cardiac muscle consists of a single fiber type, most similar to type I skeletal muscle fibers. In contrast, smooth muscle cells are not striated but rather consist of dense bodies and intermediate filaments. Smooth muscle contraction and relaxation are slower than both skeletal and cardiac muscle because contraction is accomplished by Ca^{2+}-regulated phosphorylation of myosin rather than the Ca^{2+} and troponin system. This mechanism, which can be activated by stretch, is highly efficient, requiring less ATP for contraction and allowing smooth muscle to maintain tension for prolonged periods of time with a relatively low energy cost compared with cardiac and skeletal muscles (18).

In addition to being of great importance for cellular redox homeostasis, O_2 sensing, and intracellular signaling, mitochondria are the major source of muscle ATP production (2). In fact, as the amount of ATP stored within cardiac muscle (≈10 mmol/kg) and skeletal muscle (20–25 mmol/kg) is typically insufficient to meet even the short-term dynamic energy demands of these muscles, the mitochondria are largely responsible for the continued resynthesis of this ATP (19, 25). The same is true for smooth muscle; however, due to a combination of a reduced requirement to perform work and greater efficiency of contraction, smooth muscle would be expected to contain fewer mitochondria than skeletal and cardiac muscles. Indeed, the present evaluation reveals that smooth muscle mitochondrial content, as assessed by CS activity, is only ~42% and ~22% that of skeletal and cardiac muscles, respectively. Of note, as factors that alter mitochondrial density, such as individual differences in physical activity and skeletal muscle fiber type composition, these relative mitochondrial contents may fluctuate significantly. For example, Lemieux et al. (24) previously reported that mitochondrial density in skeletal muscle was ~33% of cardiac muscle, whereas the present data revealed closer values, with skeletal muscle exhibiting ~52% of the mitochondrial density of cardiac muscle.

Interestingly, in qualitative agreement with the CS activity data and therefore mitochondrial content, the present study revealed that complex IV respiration, an indirect indicator of electron transport capacity, fell from cardiac to skeletal to smooth muscles. Consequently, CS activity and complex IV respiration were well correlated (Fig. 3*A*). Collectively, these findings confirm that electron transport capacity is dictated by mitochondrial content in skeletal and cardiac muscles but extend this finding to include smooth muscle. Additionally, the present study revealed that mitochondrial content in these three distinct muscle types is tightly linked to oxidative phosphorylation capacity (complex I + II, state 3; Fig. 2*A*) and the expected level of aerobic work based on in vivo function, with a progressive fall in capacity evident from cardiac to skeletal to smooth muscles.

Oxidative phosphorylation capacity: muscle- versus mitochondrial-specific content. Muscle-specific phosphorylation capacity (wet wt) was highest in cardiac muscle, falling progressively from skeletal to smooth muscles (Fig. 1). In terms of cardiac and skeletal muscles, these findings are well aligned with the limited studies that have revealed that the oxidative phosphorylation capacity of cardiac muscle exceeds that of skeletal muscle (24, 26) but extends these findings to include the, now documented, even lower oxidative phosphorylation capacity of smooth muscle. As would be expected, because of the strong relationship between CS activity and oxidative phosphorylation capacity (complex I + II, state 3; Fig. 2*A*), the difference in oxidative phosphorylation capacity in these three distinct tissues was negated when normalized to CS activity (Fig. 2*B*). Thus, in terms of mitochondrial content, oxidative phosphorylation capacity did not vary significantly from cardiac to skeletal to smooth muscles (Fig. 2*A*). This was also the case for the efficiency of oxidative phosphorylation capacity per mitochondrial content, which was not statistically different across the three muscle types (Fig. 3*B*).

Vessel smooth muscle mitochondrial respiration has not been comprehensively studied (15, 42, 44), with this work being not only the first to examine smooth muscle mitochondrial respiratory function with real conviction but also the first to compare human vascular smooth muscle mitochondrial respiration with that of human cardiac and skeletal muscles. Vascular smooth muscle mitochondria derived ATP production is thought to be mainly required for the maintenance of myogenic tone and facilitating cellular transport (42). In contrast, mitochondria in cardiac and skeletal muscles produce ATP to facilitate contraction and relaxation and subsequently perform considerable, measurable work. For the heart, this results in the maintenance of cardiac output and blood pressure, whereas for skeletal muscle, the task is predominantly posture and locomotion. The differing role of each of these muscles requires varied amounts of mitochondria-derived ATP production. Specifically, based on in vivo function, smooth muscle does not likely require the same level of oxidative capacity as cardiac and skeletal muscles due to the lower ATP demand required for the maintenance of myogenic tone and cellular function compared with active force generation. Thus, the reduced tissue mass-specific smooth muscle oxidative capacity, compared with both cardiac and skeletal muscles, in conjunction with a similar oxidative phosphorylation capacity per mitochondrial content, and less of a need for ATP production, teleogically seems appropriate. Therefore, although oxidative phosphorylation capacity in these three distinct muscle types is different, similar mitochondrion respiratory function but differing mitochondrial content enable each muscle tissue to match cellular energy demand with supply.

Inferences from distinct nonphosphorylating respiration and RCR across muscle types. The proton gradient between the matrix and intermembrane space of the mitochondrion (the proton motive force) facilitates ATP production, and, in combination with nonphosphorylating proton conductance (proton leak), these processes regulate the kinetics and efficiency of mitochondrial respiration (40). Proton leak also plays a role in determining the level of ROS, although it is controversial as to whether an increased proton leak reduces ROS production via decreased proton motive force and a subsequent attenuation of the coupled oxidative phosphorylation (34) or if an increased

proton leak increases O_2 consumption leading to increase ROS production (37, 41). Interestingly, in the present study, complex I state 2 respiration, an indicator of proton leak, was not different across cardiac, skeletal, and vascular smooth muscles when the respiration rate was normalized for tissue wet weight (Fig. 4*A*). This suggests that ROS production per unit of cardiac, skeletal, and smooth muscle tissues may be similar. However, when complex I state 2 respiration was normalized to CS activity, proton leak per mitochondrial content rose progressively from cardiac to skeletal to smooth muscles. This difference in proton leak per mitochondrial content between these three distinct muscle types could, potentially, be due to the different proton permeability of mitochondrial membranes in cardiac, skeletal, and smooth muscles (Fig. 4*B*) (9, 10).

In terms of ROS production and the vasculature, it should be noted that mitochondria of endothelial cells are also an important source for ROS. Recently, Ungvari and colleagues (42) suggested that increased vascular ROS production may be due to reduced mitochondrial content in endothelial cells. However, it should be noted that, in preparation for the present study, pilot data were collected in feed arteries with and without endothelium (denuded with air bubbles). Mitochondrial respiration from endothelial cells makes up an immeasurable portion of the respiration assessed with the present experimental design (state 3 respiration, complex I + II: 13 ± 5 vs. 12 ± 7 pmol·s^{-1}·mg^{-1}, respectively). This is likely due both to the very low mitochondrial content and very small overall volume of endothelial cells in the preparation. However, it is still important to acknowledge the potentially very important interaction between mitochondria-derived ROS and nitric oxide from the endothelium, which may play a significant role in the regulation of cellular respiration (29). Therefore, further investigations in this area are warranted.

The RCR, which encapsulates the main respiratory function of mitochondria inasmuch as it documents the ability to idle at a low respiratory rate but responds to ADP by making ATP at a high rate, has been suggested to be a good indicator of mitochondrial respiratory dysfunction when the electron transport chain capacity is intact (7, 8, 17). Therefore, the values of RCR in the present study can be interpreted as an index of intrinsic mitochondrial respiratory function. RCR was the highest in cardiac muscle, intermediate in skeletal muscle, and the lowest in smooth muscle (Fig. 4*C*). In this case, differing RCRs in cardiac, skeletal, and smooth muscles may be an indicator of an altered physiological role of endogenous proton leak and ADP oxidation across muscle types rather than dysfunction. This interpretation is well aligned with conclusions from previous studies that have revealed that pancreatic and liver cells have stronger control of ATP/ADP by proton leak than skeletal muscle (1, 8). Thus, based on RCR and proton leak, the present study has identified that different muscle tissues have distinct intrinsic mitochondrial respiratory functions, which likely influences the efficiency of oxidative phosphorylation and could potentially alter ROS production.

Experimental considerations. A fundamental concern in a study that examines several different muscle types, but harvests each of these muscles from different subjects, is the issue of subject heterogeneity. Indeed, the tissue used in the present study was not harvested from the same person because both logistically and ethically this would be very difficult to achieve. Therefore, it must be acknowledged that a limitation of the present study was the variability of the subjects in terms of lifestyle (e.g., diet, physical activity, etc.). However, presumably, these lifestyle differences were randomly distributed among the groups, as subjects were not selected for any particular habits. In contrast, the potential influence of anesthesia was not the same in all groups. Specifically, cardiac and smooth muscle donors were exposed to a routinely used set of anesthetics, whereas skeletal muscle donors received only topical lidocaine, and the impact of these exposures on the present results is unknown. However, the good and intuitively expected agreement between both CS activity and complex IV respiration and state 3 (compex I + II) respiration across all muscles (Figs. 2*A* and 3*A*) suggests that this was not a major confounding factor in terms of the assessment of mitochondrial respiration.

Additionally, in the present study, there were no direct measurements of redox balance or ROS, but there was speculation that ROS production may be different between the three muscles examined. This certainly warrants further studies to confirm this speculation; however, it has been well documented that increased proton leak, as documented here (Fig. 4*B*), is associated with the regulation of ROS production (34, 37, 40).

Summary. This study used the permeabilized fiber approach to assess smooth muscle mitochondrial respiration in human arteries and contrasted these novel data with findings in human cardiac and skeletal muscles. Although complex I + II, state 3 respiration rates, when normalized by CS activity, were similar in cardiac, skeletal, and smooth muscles, there were significant muscle-specific differences in both nonphosphorylating respiration and RCR. Therefore, the present study has identified that different muscle tissues have distinct intrinsic mitochondrial respiratory functions, which likely influences the efficiency of oxidative phosphorylation and could potentially alter ROS production.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: S.-Y.P., F.D., S.L., and R.S.R. conception and design of research; S.-Y.P., J.R.G., R.H.A., J.D.T., J.R.H., R.S.G., N.A.D., S.J.I., S.D., and R.S.R. performed experiments; S.-Y.P., J.R.G., R.S.G., and R.S.R. analyzed data; S.-Y.P. and R.S.R. interpreted results of experiments; S.-Y.P. prepared figures; S.-Y.P., F.D., S.L., S.D., and R.S.R. drafted manuscript; S.-Y.P., R.H.A., J.D.T., J.R.H., F.D., S.L., and R.S.R. edited and revised manuscript; R.S.R. approved final version of manuscript.

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CHAPTER 5

CONCLUSION

Conclusion

The overall purpose of this dissertation was to investigate the integration of cardiac, skeletal, and vascular smooth muscle function in terms of O_2 transport and utilization with aging and disease. Specifically, the impact of aging and disease on both function and respiratory capacity were evaluated in human muscle tissue samples utilizing *in vitro* approaches.

With the goal to better understand the age-associated reduction in vascular function in old individuals, the first study of this dissertation examined vasomotor function in human SMFAs, utilizing pressure myography. Initially, we further determined that SMFAs likely contribute to the regulation of vascular resistance/conductance *in vivo* by documenting vasodilation in response to a physiologically relevant increase in shear stress. In terms of aging, this study revealed reduced endothelial function in the elderly, which was identified by attenuated vasodilation kinetics in old SMFAs compared to young. Additionally, maximal endothelium-mediated vasodilatory capacity in response to physiological stimuli, increased shear stress, and pharmacological stimuli, Ach dose response, was significantly reduced. These findings suggest that the attenuated endothelial function observed with aging in human SMFAs may be a contributing factor to impaired O_2 delivery capacity with advancing age. Also, the attenuated vascular function with age was associated with a reduced shear-induced activation of eNOS, and elevated ROS production in human SMFAs. Therefore, the present study has identified blunted endothelial function and elevated free radicals in human SMFAs with age and these deleterious changes may contribute to the exercise intolerance commonly observed with advancing aging age, by diminishing O_2 transport.

The second study of this dissertation sought to examine the impact of HF etiology on mitochondrial function in HF. Specifically, cardiac muscle tissue mitochondrial function and free radical levels were evaluated in cardiac muscle from patients with iHF and niHF in comparison to those from healthy donors hearts (HdH). Both mitochondrial quality and quantity were compromised in HF compared to HdH. Interestingly, however, tissue mass-specific OXPHOS was even lower in iHF compared to niHF but mitochondrial content was also significantly lower in iHF compared to niHF. Thus, when tissue mass-specific OXPHOS capacity was normalized for mitochondrial content, the difference in tissue mass-specific OXPHOS between iHF and niHF was negated. Interestingly, iHF exhibited greater non-phosphorylating respiration, and a lower OXPHOS efficiency compared to niHF. Furthermore, total free radical levels and mitochondrial-derived superoxide levels were elevated in iHF compared to niHF. Collectively, these data suggest an etiology-specific reduction in intrinsic mitochondrial function. Therefore, the second study of this dissertation has identified that HF etiology plays an important role in the mitochondrial dysfunction in HF. These findings suggest that therapeutically targeting mitochondrial content, as well as mitochondrial free radical production, potentially in an etiology- specific manner, could result in progress in treating patients with HF.

The third study of this dissertation examined the characteristics and respiratory function of mitochondria in cardiac, skeletal, and vascular smooth muscle by assessing mitochondrial respiration in the smooth muscle of human SMFAs, in comparison to that of cardiac and skeletal muscle. This study documented the ability to successfully measure vascular smooth muscle mitochondrial respiration using the permeablized fiber approach

routinely used with both cardiac and skeletal muscles. Within the tissue types, tissue mass-specific mitochondrial OXPHOS fell from cardiac to skeletal, to smooth muscle. Also, mitochondrial content as assessed by citrate synthase activity (CSA), fell progressively from cardiac, to skeletal, to smooth muscle. Therefore, when this tissue mass-specific OXPHOS was normalized by CSA, the differences in OXPHOS capacity were no longer apparent. Interestingly, there were significant muscle-specific differences in both non-phosphorylating respiration and the OXPHOS efficiency. These findings document that different muscle types have distinct intrinsic mitochondrial function, which appear to affect the OXPHOS efficiency and therefore may potentially impact free radical production.

In summary, this research examined the integration of cardiac, skeletal, and vessel smooth muscle in O_2 transport and utilization with aging and disease. Specifically, this dissertation research has identified that aging and disease attenuate O_2 transport and utilization by adversely impacting vascular endothelial and mitochondrial function, which are both associated with elevated oxidative stress. Importantly, these maladaptations may be therapeutic targets for improving impaired O_2 transport and utilization with aging and disease. Therefore, by studying the components and integrative function of these three muscle systems, this research may contribute significantly to our understanding of the limitations to O_2 transport and utilization with aging and disease**.** Collectively, the conclusions gathered from this dissertation provide mechanistic insight which may be utilized to improve functional capacity with aging and disease, and therefore potentially enhance the quality of life in old and diseased populations.